Conclusion

This initial experience in lupus patients with mild to moderate symptoms demonstrated that 4 doses of 360 mg/m^2 epratuzumab immunotherapy are safe and well tolerated when infused within one hour, with consistent improvement observed in all patients for at least 12 weeks in the presence of modestly decreased (about 35%) peripheral B cell levels, and with no evidence of HAHA. Although this was an open-label study, consistent improvement was observed in all patients for at least 12 weeks, and there was reduction or elimination of disease activity across most body systems, regardless of the extent or the severity of the presenting disease activity. The duration of response was very heterogeneous for different BILAG domains, precluding firm conclusions at this time. As such, these results support conducting longer-term multicenter randomized controlled studies, which are now underway to examine the effects of epratuzumab in broader patient populations with autoimmune disease.

Competing interests

TD, JK, and GRB declare research funding for this study provided by Immunomedics, Inc. WAW, NT, and DMG have employment and financial interests (stock) in Immunomedics, Inc., whichowns the antibody tested in this paper.

Authors' contributions

All authors contributed to data interpretation and the final manuscript. TD and GRB were the principal investigators and were responsible for coordinating the study, while JK participated in patient selection and directed all patient related study procedures. DMG, TD and WAW designed the clinical trial protocol, and NT was responsible for data management and statistical analysis. TD and JK contributed equally to this work.

Acknowledgements

The authors acknowledge the patients who agreed to participate in this study. This study was supported in part by the Sonderforschungsbereich 650 (TD, GRB), and by Immunomedics, Inc. We thank Vibeke Strand, MD, for her helpful comments for improving the manuscript.

References

1. Snaith ML, Isenberg DA: **Systemic lupus erythematosus and related disorders.** In *Oxford Textbook of Medicine* 3rd edition. Edited by: Weatherall DJ, Ledingham JGG, Warrell DA. Oxford: Oxford University Press; 1996:3017-3027.
2. Jacobson DL, Gange SJ, Rose NR, Graham NM: **Epidemiology and estimated population burden of selected autoimmune diseases in the United States.** *Clin Immunol Immunopathol* 1997, **84**:223-243.
3. Datta SK: **Production of pathogenic antibodies: cognate interactions between autoimmune T and B cells.** *Lupus* 1998, **7**:591-596.
4. Llorente L, Richaud-Patin Y, Wijdenes J, Alcocer-Varela J, Maillot MC, Durand-Gasselin I, Fourrier BM, Galanaud P, Emilie D: **Spontaneous production of interleukin-10 by B lymphocytes and monocytes in systemic lupus erythematosus.** *Eur Cytokine Netw* 1993, **4**:421-427.
5. Jacobi AM, Odendahl M, Reiter K, Bruns A, Burmester GR, Radbruch A, Valet G, Lipsky PE, Dörner T: **Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus.** *Arthritis Rheum* 2003, **48**:1332-1342.
6. Looney RJ, Anolik JH, Campbell D, Felgar RE, Young F, Arend LJ, Sloand JA, Rosenblatt J, Sanz I: **B cell depletion as a novel treatment for systemic lupus erythematosus: a phase I/II dose-escalation trial of rituximab.** *Arthritis Rheum* 2004, **50**:2580-2589.
7. Sfikakis PP, Boletis JN, Tsokos GC: **Rituximab anti-B cell therapy in systemic lupus erythematosus: pointing to the future.** *Curr Opin Rheumatol* 2005, **17**:550-557.
8. Rajagopalan S, Zordan T, Tsokos GC, Datta SK: **Pathogenic anti-DNA autoantibody-inducing T helper cell lines from patients with active lupus nephritis: isolation of CD4-8- T helper cell lines that express the gamma delta T-cell antigen receptor.** *Proc Natl Acad Sci USA* 1990, **87**:7020-7024.9.
9. de Vos AF, Fukushima A, Lobanoff MC, Vistica BP, Lai JC, Grivel JC, Wawrousek EF, Whitcup SM, Gery I: **Breakdown of tolerance to a neo-self antigen in double transgenic mice in which B cells present the antigen.** *J Immunol* 2000, **164**:4594-4600.
10. Roth R, Gee RJ, Mamula MJ: **B lymphocytes as autoantigen-presenting cells in the amplification of autoimmunity.** *Ann NY Acad Sci* 1997, **815**:88-104.
11. Demaison C, Chastagner P, Theze J, Zouali M: **Somatic diversification in the heavy chain variable region genes expressed by human autoantibodies bearing a lupus-associated nephritogenic anti-DNA idiotype.** *Proc Natl Acad Sci USA* 1994, **91**:514-518.
12. Manheimer-Lory AJ, Zandman-Goddard G, Davidson A, Aranow C, Diamond B: **Lupus-specific antibodies reveal an altered pattern of somatic mutation.** *J Clin Invest* 1997, **100**:2538-2546.
13. Grammer AC, Slota R, Fischer R, Gur H, Girschick H, Yarboro C, Illei GG, Lipsky PE: **Abnormal germinal center reactions in systemic lupus erythematosus demonstrated by blockade of CD154-CD40 interactions.** *J Clin Invest* 2003, **112**:1506-1520.
14. Andrews BS, Eisenberg RA, Theofilopoulos AN, Izui S, Wilson CB, McConahey PJ, Murphy ED, Roths JB, Dixon FJ: **Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains.** *J Exp Med* 1978, **148**:1198-1215.
15. Shlomchik MJ, Madaio MP, Ni D, Trounstein M, Huszar D: **The role of B cells in lpr/lpr-induced autoimmunity.** *J Exp Med* 1994, **180**:1295-1306.
16. Chan O, Shlomchik MJ: **A new role for B cells in systemic autoimmunity: B cells promote spontaneous T cell activation in MRL-lpr/lpr mice.** *J Immunol* 1998, **160**:51-59.
17. Chan OT, Hannum LG, Haberman AM, Madaio MP, Shlomchik MJ: **A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus.** *J Exp Med* 1999, **189**:1639-1648.
18. Dörner T, Radbruch A: **Selecting B cells and plasma cells to memory.** *J Exp Med* 2005, **201**:497-499.
19. Leandro MJ, Edwards JC, Cambridge G, Ehrenstein MR, Isenberg DA: **An open study of B lymphocyte depletion in systemic lupus erythematosus.** *Arthritis Rheum* 2002, **46**:2673-2677.
20. Eisenberg R, Albert D, Stansberry J, Tsai D, Kolasinski S, Khan S: **A phase I trial of B cell depletion with anti-CD20 monoclonal antibody (rituximab) in the treatment of systemic lupus erythematosus [abstract].** *Arthritis Res Ther* 2003, **5(Suppl 3)**:S9-10.
21. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowka A, Emery P, Close DR, Stevens RM, Shaw T: **Efficacy of B cell-targeted therapy with rituximab in patients with rheumatoid arthritis.** *N Engl J Med* 2004, **350**:2572-2581.
22. Van Vollenhoven RF, Gunnarsson I, Welin-Henriksson E, Sundelin B, Jacobson SH, Kareskog L: **A 4-week course of rituximab plus cyclophosphamide in severe SLE: promising results in 9 patients who failed conventional immunosuppressive therapy. EULAR 2004 [abstract].** *Ann Rheum Dis* **63(Suppl 1)**:863HH.
23. Tedder TF, Tuscano J, Sato S, Kehrl JH: **CD22, a B lymphocyte-specific adhesion molecule that regulates antigen receptor signaling.** *Annu Rev Immunol* 1997, **15**:481-504.
24. Peaker CJ, Neuberger MS: **Association of CD22 with the B cell antigen receptor.** *Eur J Immunol* 1993, **23**:1358-1363.
25. Nath D, van der Merwe PA, Kelm S, Bradfield P, Crocker PR: **The amino-terminal immunoglobulin-like domain of sialoadhesin contains the sialic acid binding site. Comparison with CD22.** *J Biol Chem* 1995, **270**:26184-26191.

26. Sgroi D, Koretzky GA, Stamenkovic I: **Regulation of CD45 engagement by the B cell receptor CD22.** *Proc Natl Acad Sci USA* 1995, **92**:4026-4030.
27. Kelm S, Pelz A, Schauer R, Filbin MT, Tang S, de Bellard ME, Schnaar RL, Mahoney JA, Hartnell A, Bradfield P, *et al.*: **Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily.** *Curr Biol* 1994, **4**:965-972.
28. Clark EA: **CD22, a B cell-specific receptor, mediates adhesion and signal transduction.** *J Immunol* 1993, **150**:4715-4718.
29. Engel P, Nojima Y, Rothstein D, Zhou LJ, Wilson GL, Kehrl JH, Tedder TF: **The same epitope on CD22 of B lymphocytes mediates the adhesion of erythrocytes, T and B lymphocytes, neutrophils, and monocytes.** *J Immunol* 1993, **150**:4719-4732.
30. Powell LD, Varki A: **I-type lectins.** *J Biol Chem* 1995, **270**:14243-14246.
31. Otipoby KL, Andersson KB, Draves KE, Klaus SJ, Farr AG, Kerner JD, Perlmutter RM, Law CL, Clark EA: **CD22 regulates thymus-independent responses and the lifespan of B cells.** *Nature* 1996, **384**:634-637.
32. Poe JC, Fujimoto M, Jansen PJ, Miller AS, Tedder TF: **CD22 forms a quaternary complex with SHIP, Grb2, and Shc. A pathway for regulation of B lymphocyte antigen receptor-induced calcium flux.** *J Biol Chem* 2000, **275**:17420-17427.
33. O'Keefe TL, Williams GT, Batista FD, Neuberger MS: **Deficiency in CD22, a B cell-specific inhibitory receptor, is sufficient to predispose to development of high affinity autoantibodies.** *J Exp Med* 1999, **189**:1307-1313.
34. Sato S, Tuscano JM, Inaoki M, Tedder TF: **CD22 negatively and positively regulates signal transduction through the B lymphocyte antigen receptor.** *Semin Immunol* 1998, **10**:287-297.
35. Nadler MJ, McLean PA, Neel BG, Wortis HH: **B cell antigen receptor-evoked calcium influx is enhanced in CD22-deficient B cell lines.** *J Immunol* 1997, **159**:4233-4243.
36. Nitschke L, Carsetti R, Ocker B, Kohler G, Lamers MC: **CD22 is a negative regulator of B cell receptor signaling.** *Curr Biol* 1997, **7**:133-143.
37. Chen J, McLean PA, Neel BG, Okunade G, Shull GE, Wortis HH: **CD22 attenuates calcium signaling by potentiating plasma membrane calcium-ATPase activity.** *Nat Immuno* 2004, **5**:651-657.
38. Pawlak-Byczkowska EJ, Hansen HJ, Dion AS, Goldenberg DM: **Two new monoclonal antibodies, EPB-1 and EPB-2, reactive with human lymphoma.** *Cancer Res* 1989, **49**:4568-4577.
39. Stein R, Belisle E, Hansen HJ, Goldenberg DM: **Epitope specificity of the anti-(B cell lymphoma) monoclonal antibody, LL2.** *Cancer Immunol Immunother* 1993, **37**:293-298.
40. Leung SO, Goldenberg DM, Dion AS, Pellegrini MC, Shevitz J, Shih LB, Hansen HJ: **Construction and characterization of a humanized, internalizing, B cell (CD22)-specific, leukemia/lymphoma antibody, LL2.** *Mol Immunol* 1995, **32**:1413-1427.
41. Gada P, Hernandez-Ilizaliturri F, Repasky EA, Czuczman MS: **Epratuzumab's predominant antitumor activity *in vitro/in vivo* against non-Hodgkin's lymphoma (NHL) is via antibody-dependent cellular cytotoxicity (ADCC) [abstract].** *Blood* 2002, **100/11**:353a.
42. Leonard JP, Coleman M, Ketas JC, Chadburn A, Ely S, Furman RR, Wegener WA, Hansen HJ, Ziccardi H, Eschenberg M, *et al.*: **Phase I/II trial of epratuzumab (humanized anti-CD22 antibody) in indolent non-Hodgkin's lymphoma.** *J Clin Oncol* 2003, **21**:3051-3059.
43. Leonard JP, Coleman M, Ketas JC, Chadburn A, Furman R, Schuster MW, Feldman EJ, Ashe M, Schuster SJ, Wegener WA, *et al.*: **Epratuzumab, a humanized anti-CD22 antibody, in aggressive non-Hodgkin's lymphoma: phase I/II clinical trial results.** *Clin Cancer Res* 2004, **10**:5327-5334.
44. Perotti B, Doshi S, Chen D, Gayko U, Leonard JP, Wegener WA, Goldenberg DM, Cesano A: **Pharmacokinetics of epratuzumab administered as a single agent or in combination with rituximab in patients with B cell NHL [abstract].** *Proc Am Soc Clin Oncol* 2003, **22**:.
45. Hay EM, Bacon PA, Gordon C, Isenberg DA, Maddison P, Snaith ML, Symmons DPM, Viner N, Zoma A: **The BILAG index: a reliable and valid instrument for measuring clinical disease activity in systemic lupus erythematosus.** *Quart J Med* 1993, **86**:447-458.
46. Isenberg DA, Gordon C: **From BILAG to BLIPS. Disease activity assessment in lupus: past, present and future.** *Lupus* 2000, **9**:651-654.
47. Odendahl M, Jacobi A, Hansen A, Feist E, Hiepe F, Burmester GR, Lipsky PE, Radbruch A, Dörner T: **Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus.** *J Immunol* 2000, **165**:5970-5979.
48. Silverman GJ: **Anti-CD20 therapy in systemic lupus erythematosus: a step closer to the clinic.** *Arthritis Rheum* 2005, **52**:371-377.
49. Carnahan J, Wang P, Kendall R, Chen C, Hu S, Boone T, Juan T, Talvenheimo J, Montestruque S, Sun J, *et al.*: **Epratuzumab, a humanized monoclonal antibody targeting CD22: characterization of *in vitro* properties.** *Clin Cancer Res* 2003, **9**:3982s-3990s.

Research article

Open Access

Epratuzumab (humanised anti-CD22 antibody) in primary Sjögren's syndrome: an open-label phase I/II study

Serge D Steinfeld[1], Laure Tant[1], Gerd R Burmester[2], Nick KW Teoh[3], William A Wegener[3], David M Goldenberg[3] and Olivier Pradier[4]

[1]Department of Rheumatology, Erasme University Hospital, 808 Route de Lennik, Brussels 1070, Belgium
[2]Department of Rheumatology, Charite Hospital, Schumannstr 20-21, Berlin D-10098, Germany
[3]Immunomedics, Inc., Morris Plains, 300 American Road, New Jersey 07950, USA
[4]Laboratory of Hematology, Erasme University Hospital, 808 Route de Lennik, Brussels 1070, Belgium

Corresponding author: Serge D Steinfeld, ssteinfe@ulb.ac.be

Received: 9 May 2006 Revisions requested: 8 Jun 2006 Revisions received: 16 Jun 2006 Accepted: 20 Jul 2006 Published: 20 Jul 2006

Arthritis Research & Therapy 2006, 8:R129 (doi:10.1186/ar2018)
This article is online at: http://arthritis-research.com/content/8/4/R129

Abstract

This open-label, phase I/II study investigated the safety and efficacy of epratuzumab, a humanised anti-CD22 monoclonal antibody, in the treatment of patients with active primary Sjögren's syndrome (pSS). Sixteen Caucasian patients (14 females/2 males, 33–72 years) were to receive 4 infusions of 360 mg/m^2 epratuzumab once every 2 weeks, with 6 months of follow-up. A composite endpoint involving the Schirmer-I test, unstimulated whole salivary flow, fatigue, erythrocyte sedimentation rate (ESR), and immunoglobulin G (IgG) was devised to provide a clinically meaningful assessment of response, defined as a ≥20% improvement in at least two of the aforementioned parameters, with ≥20% reduction in ESR and/or IgG considered as a single combined criterion. Fourteen patients received all infusions without significant reactions, 1 patient received 3, and another was discontinued due to a mild acute reaction after receiving a partial infusion. Three patients showed moderately elevated levels of Human anti-human (epratuzumab) antibody not associated with clinical manifestations. B-cell levels had mean reductions of 54% and 39% at 6 and 18 weeks, respectively, but T-cell levels, immunoglobulins, and routine safety laboratory tests did not change significantly. Fifty-three percent achieved a clinical response (at ≥20% improvement level) at 6 weeks, with 53%, 47%, and 67% responding at 10, 18, and 32 weeks, respectively. Approximately 40%–50% responded at the ≥30% level, while 10%–45% responded at the ≥50% level for 10–32 weeks. Additionally, statistically significant improvements were observed in fatigue, and patient and physician global assessments. Further, we determined that pSS patients have a CD22 over-expression in their peripheral B cells, which was downregulated by epratuzumab for at least 12 weeks after the therapy. Thus, epratuzumab appears to be a promising therapy in active pSS, suggesting that further studies be conducted.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease with a population prevalence of approximately 0.5% [1]. The lymphoid infiltrates within the inflamed tissues contain ectopic germinal center-like structures in 20% of patients [2]. These structures consist of T- and B-cell aggregates containing proliferating lymphocytes, follicular, dendritic, and activated endothelial cells [3]. B-cell homeostasis is disturbed in pSS, with diminished frequencies and absolute numbers of peripheral CD27$^+$ memory B cells. However, the infiltrating B cells are mainly CD27$^+$ memory B cells and CD27^{high} plasma cells [4-6]. This altered B-cell subtype recirculation from inflamed tissue was confirmed recently by Hansen *et al.* [7]. Thus, although pSS is considered to be a T-cell-mediated disease, high levels of B-cell autoreactivity have been associated with high disease activity, the development of systemic com-

ACR = American College of Rheumatology; AE = adverse event; ANA = antinuclear antibody; APC = allophycocyanin; AT = artificial tear; BCR = B-cell antigen receptor; CRP = C-reactive protein; CTC = [National Cancer Institute] Common Toxicity Criteria; ELISA = enzyme-linked immunosorbent assay; ESR = erythrocyte sedimentation rate; HACA = human anti-chimeric antibody; HAHA = human anti-human (epratuzumab) antibody; Ig = immunoglobulin; NHL = non-Hodgkin's lymphoma; PE = phycoerythrin; pSS = primary Sjögren's syndrome; RF = rheumatoid factor; SD = standard deviation; SLE = systemic lupus erythematosus; USF = unstimulated whole salivary flow; VAS = visual analogue scale.

plications, and an increased risk of development of B-cell lymphoma [8].

This has led to anti-B-cell monoclonal antibody immunotherapy emerging as a promising new treatment modality in pSS and autoimmune disorders [9]. The use of rituximab, a chimeric anti-CD20 antibody, has been reported in small studies and case reports of SS patients with or without associated lymphoma [10-13]. However, serum sickness-like diseases seem to occur in approximately 20% of patients treated with this chimeric antibody [11] and may be of major clinical concern in subjects with a hyperactive immune system.

CD22 is a 135-kDa B-lymphocyte restricted type-I transmembrane sialoglycoprotein of the immunoglobulin (Ig) superfamily, with seven Ig-like domains and three cytoplasmic ITIMs (immunoreceptor tyrosine-based inhibitory motifs) [14]. CD22 appears intracellularly during the late pro-B-cell stage of ontogeny, shifting to the plasma membrane with B-cell maturation. CD22 is expressed at low levels on immature B cells, expressed at higher levels on mature IgM^+, IgD^+ B cells, and absent on differentiated plasma cells. It is strongly expressed in follicular, mantle, and marginal-zone B cells but is weakly present in germinal B cells (reviewed in [15]). The function of CD22 has not been entirely clarified; it acts as a homing receptor for recirculating B cells through the affinity of the lectin-like domains for 2,6-linked sialic acid-bearing glycans and as a B-cell antigen receptor (BCR) down-modulating coreceptor [16,17]. Because dysregulated expression of CD22 could lead to excessive activation of B cells and autoantibody production [17], targeting this coreceptor in systemic autoimmunity appears to be a potentially new therapeutic pathway. Indeed, antagonistic antibodies to CD22 could provoke downregulation of the BCR (by SHP-1 [Src homology phosphatase-1] recruitment) and inhibiting T- and B-cell crosstalk by downregulation of the CD40 pathway [15,18,19].

Epratuzumab (hLL2), a humanised IgG1 monoclonal antibody directed against the CD22 antigen, binds the third cytoplasmic Ig domain of CD22 [20]. Its known mechanism of action is believed to be the downregulation of the BCR, with mechanisms of action differing from rituximab (by CD22 phosphorylation and BCR effects via immobilised Ig crosslinking) [18,21]. Also, epratuzumab depletes circulating B cells when given to patients with non-Hodgkin's lymphoma (NHL) [22] or systemic lupus erythematosus (SLE) [23], but markedly less than rituximab [24], and has been therapeutically active in both diseases.

Based on these considerations, this phase I/II study was undertaken to investigate the safety and potential local and systemic effects of epratuzumab in patients with active pSS. The effect of this treatment on the peripheral B and T cells in this patient population was also assessed.

Materials and methods

Study design

This phase I-II, open-label, 18-week study was conducted at two centres. The protocol was approved by the local ethics committees, and all patients enrolled were required to provide signed informed consent. The study included four infusions of epratuzumab (360 mg/m^2) at 0, 2, 4, and 6 weeks and three follow-up evaluations at 6, 10, and 18 weeks (that is, 1 day, 4 weeks, and 12 weeks after the fourth infusion). Additionally, a final long-term evaluation was scheduled at 32 weeks (6 months post-treatment).

Patient population

Males or non-pregnant, non-lactating females, at least 18 years of age, were eligible to participate provided that they fulfilled the American/European consensus group classification criteria for pSS [25]. For females of childbearing potential, a negative pregnancy test result and adequate contraception during the study and for 6 months after the last infusion were required. Additionally, all patients had to demonstrate active pSS prior to study entry. Because there are no validated disease activity criteria for pSS, this was defined as increased B-cell activity, IgG greater than 1.4 g/l or erythrocyte sedimentation rate (ESR) greater than 25 mm/hour, in combination with the presence of autoantibodies. In addition, the patients must have been on symptomatic treatment for at least 6 months prior to screening. Disease-modifying drugs, such as hydroxychloroquine, methotrexate, cyclosporin, sulfasalazine, or corticosteroids, were not allowed during the study and were discontinued at least 4 weeks before study entry. No prior treatment with rituximab or other anti-B-cell antibodies was allowed. The exclusion criteria included serious infections in the previous 3 months, documented HIV or hepatitis-B or -C infection, known malignancy, severe or uncontrolled concurrent disease, and the presence of any other autoimmune/connective tissue disease.

Study drug administration

Epratuzumab at the dose of 360 mg/m^2 in 250 ml 0.9% sterile NaCl was prepared by the hospital pharmacy (Erasme University Hospital and Charite Humboldt University Hospital). The total dose was to be given throughout a 40-minute period. To minimise hypersensitivity, patients were premedicated with acetaminophen (0.5–1 g) and antihistamine (25–50 mg *per os* or intravenous polaramin). Four intravenous infusions of epratuzumab were given at 0, 2, 4, and 6 weeks.

Concomitant medications

Patients were allowed to continue artificial tears (ATs) and artificial saliva substitutes or nonsteroidal anti-inflammatory drugs provided that the dosage and schedule regimens were stable for at least 4 weeks and were monitored during the study.

Clinical assessment

Clinical, ophthalmological, and biological evaluations were performed at study entry and at 6, 10, and 18 weeks (that is, 1 day, 4 weeks, and 12 weeks post-treatment). A final evaluation at 32 weeks was also scheduled for patients who were still in long-term follow-up. Clinical assessment (performed by the same physician) consisted of a general physical examination; a dry mouth evaluation (0–2 scale: 0 = none, 1 = mild to moderate, and 2 = severe) involving the collection of unstimulated whole saliva throughout a 15-minute interval by using the spitting technique according to established methods [26] (note: saliva samples were weighed on an analytical balance to determine the volume of saliva obtained, using the conversion formula 1 g = 1 ml); a dry eye evaluation (0–2 scale: 0 = no symptoms, 1 = mild to moderate symptoms relieved by ATs, and 2 = severe symptoms unrelieved by AT); the Schirmer-I test; evaluation of fatigue by a 0–100 mm visual analogue scale(VAS) and a questionnaire (0 = no fatigue, 1 = mild fatigue not interfering with daily activities, 2 = moderate fatigue that interferes with daily activities, and 3 = fatigue with severely reduced activities); the tender/swollen joint count (maximum 36); and the tender point count (maximum 18). The patient's pain assessment and the patient's and physician's global assessments were evaluated by a 0–100 mm VAS. The following biological parameters were measured throughout the study: ESR, C-reactive protein (CRP), complete blood count, renal and liver function tests, creatine phosphokinase, serum Igs (A, M, and G), antinuclear antibodies (ANAs), rheumatoid factor (RF), and peripheral blood B- and T-cell counts.

For purposes of efficacy evaluation, we focused on the main parameters that consisted of the the following parameters: Schirmer-I test, unstimulated whole salivary flow (USF), fatigue VAS, and the laboratory parameters (ESR and IgG). To assess the overall efficacy of epratuzumab in the treatment of pSS, a composite endpoint involving all five parameters was devised to provide a clinically meaningful definition of response. Specifically, a patient was deemed to be a responder if he/she experienced improvement of 20% or more in at least two of the aforementioned parameters, with reduction of at least 20% in ESR and/or IgG contributing jointly as a single combined criterion. Additional assessments of the efficacy data were also performed using improvements of at least 30% and at least 50% in the efficacy parameters.

Determination of B-cell populations by flow cytometry

Monoclonal antibodies used in this study were CD19 allophycocyanin (APC) (clone SJ25C1) and CD22 phycoerythrin (PE) (clone S-HCL-1). The median channel of fluorescence on the 256-channel linear scale was employed to define numerically the fluorescence distribution of CD22 in a semi-quantitative approach. We routinely used a four-color FACScalibur with automatic loader, driven by Cellquest software that was set up using the three-color FACScomp software and Calibrite microbeads (Becton, Dickinson and Company, San Jose, CA, USA). The stability of the fluorescence intensity signal over a long period of time was assessed using Quantum 1000 microbeads (weekly) and daily with the Rainbow calibration beads from Spherotec (Libertyville, IL, USA) without changing the PMT (photomultiplier tube) voltage and compensations. At the beginning of each new lot of beads, we determined an acceptable range by running aliquots of all beads 10 times and calculating the mean ± standard deviation (SD) and the CV (coefficient of variation) for that lot of beads. For each lot, we determined a mean target channel value for monitoring of flow cytometer performance. Between lots, flow cytometer settings were adjusted to restart the monitoring with the same target channel value as before. In a previous study with 35 hematological patients sampled more than three times during their clinical course (with a mean inter-visit interval of 123 days and range of 13–638 days) and a mean duration of the survey of 492 days (range 13–1,022 days), we observed an inter-contact variation of only 6.2% for CD5 (which has a sharp distribution in CLL [chronic lymphocytic leukemia]) and 6.1% for CD20. Considering the low scattering of CD22 expression on normal B-lymphocytes, we determined a range of normal values and the median CD22 fluorescence on 33 blood samples from normal healthy volunteers. Because the distribution of the median intensity is normal, we determined ± 2 SD of the distribution to define the normal median fluorescence values from a normal range between channels 159 and 178.

Tri-color immunophenotyping of B-lymphocytes was performed with predetermined combinations of murine monoclonal or rabbit polyclonal (for the Ig light chain staining) antibodies directly conjugated with fluorescein isothiocyanate, PE, and CD19 APC as a marker for B-lymphocytes. A lysed and washed whole-blood technique was used. In this procedure, 50 μl blood samples were incubated with the Mab combination at room temperature for 15 minutes. The red blood cells were then lysed using 500 μl ammonium chloride lysing solution. Cells gated in the mononuclear area in a forward-versus side-scatter dot-plot and also those present in a region around the CD19-positive side-scatter low events were considered to be B cells.

Safety assessments

During the infusion and for 1 hour afterward, the patients were monitored for adverse reactions and vital signs (blood pressure, pulse, and temperature) every 30 minutes. At each visit, patients were asked about any adverse events (AEs) that they experienced. Analysis of pharmacokinetics consisted of epratuzumab levels measured at 30 minutes prior to and after each infusion and at 6, 8, 9, 10, 14, and 18 weeks. Human anti-human (epratuzumab) antibodies (HAHAs) were assessed at study entry and at 6, 10, and 18 weeks.

Determination of anti-Ro and anti-La autoantibodies

An indirect immunofluorescence procedure using HEp-2000 cells was employed to detect the presence and titer of ANA

Table 1

Patient demographics and baseline disease characteristics

Parameters	*n* = 15
Gender (female/male)	13/2
Age (years)	49 (33–73)
Median years post-diagnosis	2.9 (1–16)
Ocular symptoms	15 (100%)
Schirmer-I test (mm)	12 ± 12
Oral symptoms	15 (100%)
Unstimulated salivary flow (ml/minute)	0.07 ± 0.13
Moderate-to-severe fatigue	13 (87%)
Fatigue VAS (mm)	56 ± 22
Focus score ≥ 1	12 (80%)
Anti-Ro antibodies	12 (80%)
Anti-La antibodies	11 (73%)
ESR (mm/hour)	33 ± 15
IgG (mg/dl)	2,114 ± 934

ESR, erythrocyte sedimentation rate; Ig, immunoglobulin; VAS, visual analogue scale.

(Immunoconcept, Sacramento, CA, USA). Anti-Ro/SS-A and anti-La/SS-B antibodies were detected both by fluoro-enzymo-immuno assay (Phadia AB, Uppsala, Sweden) and homemade double immunodiffusion. The serum levels of RF were evaluated by laser nephelometry (N Latex RF; Dade Behring, Inc., Deerfield, IL, USA). Those evaluations were performed at study entry and at 6, 10, and 18 weeks (that is, 1 day, 4 weeks, and 12 weeks post-treatment).

HAHA assay

The sponsor's [Immunomedics, Inc., Morris Plains, New Jersey, USA] HAHA test is a competitive enzyme-linked immunosorbent assay (ELISA) in which the capture reagent is epratuzumab and the probe is an anti-epratuzumab-idiotype antibody. The anti-idiotype antibody is an acceptable surrogate for what is reacted against in an immunogenic response by humans against the binding portion of epratuzumab which distinguishes the molecule from other human antibodies (that is, the framework region that has human amino acid sequences). Test results are derived from an eight-point standard curve with varying dilutions of anti-idiotype antibody in bovine serum albumin. Patient serum samples are diluted 1:2 with bovine serum albumin and assayed in triplicate. The anti-idiotype standard curve is used to determine the presence of HAHA in unknown samples. An acceptable assay is based on linear regression parameters that must be met to define a valid assay.

Statistical analysis

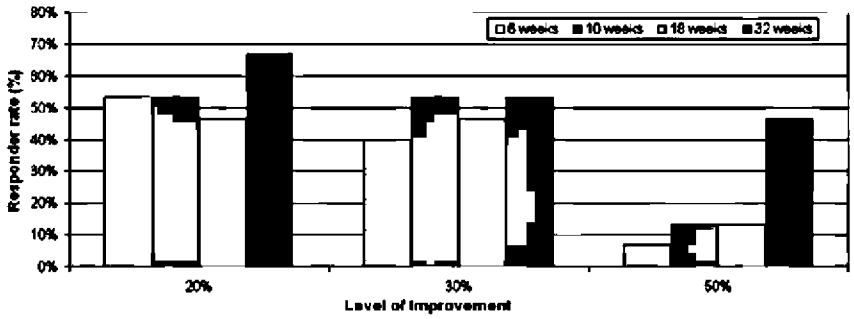
In general, discrete variables, including responder rates and AEs, were summarised using frequency counts and percentages. Percentage changes in individual efficacy parameters, B- and T-cell counts, Igs, duration of infusion times, and other continuous numerical variables were summarised using descriptive statistics. The Wilcoxon signed rank test was used to assess the statistical significance of changes in the subjective efficacy measures (VAS scores), tender points, tender joints, ESR, CRP, B cells, T cells, and Igs, compared with their baseline values. All statistical tests used a significance level of ≤0.05.

Results

Demographics and baseline disease characteristics

Sixteen patients who met the inclusion/exclusion criteria were to receive four infusions of 360 mg/m^2 epratuzumab once every 2 weeks. One patient was discontinued from the study upon receiving a partial dose of the first infusion due to a moderate acute reaction (considered as serious due to a mandatory brief overnight hospitalisation for observation). The ensuing discussion, except for safety, will focus on 15 patients who received at least one infusion of the study drug and had at least one follow-up evaluation. Their baseline characteristics are summarised in Table 1. Among the more notable medical histories, seven patients had pulmonary involvement, five had parotid enlargement, five had thyroid disease, four had neurological impairment, two had Raynaud's disease, and one had type-II cryoglobulinemia.

Figure 1



Responder rates. The overall response of a patient was determined using the four domains: dryness of the eyes (Shirmer-I test), dryness of the mouth (unstimulated whole salivary flow), fatigue (visual analogue scale), and laboratory (erythrocyte sedimentation rate and/or immunoglobulin G). A patient who achieved at least 20% improvement in at least two domains is considered a responder.

Efficacy

Fourteen patients completed the study through the 18-week (12 weeks post-treatment) evaluation period, although one among these missed the 10-week (4 weeks post-treatment) visit. The remaining patient did not receive the fourth infusion but subsequently returned for evaluation at 6 weeks. A total of 10 patients returned for the final evaluation at 32 weeks (6 months post-treatment).

General response

To assess the overall efficacy of epratuzumab in the treatment of pSS, a composite endpoint involving the Schirmer-I test, USF, fatigue VAS, and the laboratory parameters (ESR and IgG) was devised to provide a clinically meaningful definition of response, as defined in Materials and methods. Of all patients who received at least one dose of the study drug (n = 15) (Figure 1), more than half (53%) experienced a clinically meaningful response of improvement of 20% or more in two out of the four criteria at the first post-treatment evaluation at 6 weeks (24 hours after the fourth infusion). The same level of response was maintained through 10 weeks and decreased only slightly at 18 weeks (47%). At the final 32-week evaluation, 67% of the patients still showed a response that satisfied the response criteria. The corresponding results, when the response rates are calculated based on only available patients, are 53%, 62%, 50%, and 91% at 6, 10, 18, and 32 weeks, respectively.

The corresponding response rates based on improvements of at least 30% and 50% also are shown in Figure 1. Remarkably, approximately 50% of the patients also achieved the stricter responder criteria of improvement of at least 30% in two of four efficacy parameters by 10 weeks and continued to do so through 32 weeks. The corresponding response rates based on improvement levels of at least 50% were 10%–45%, depending on the visit.

Figure 2

Improvement rates in individual efficacy parameters. The improvement rate is based on achieving an improvement of at least 20% from baseline. ESR, erythrocyte sedimentation rate; IgG, immunoglobulin G; VAS, visual analogue scale.

Table 2

Subjective measures assessed by visual analogue scale (0–100 mm)

Parameters	Baseline ($n = 16$)	6 weeks ($n = 15$)	10 weeks ($n = 13$)	18 weeks ($n = 14$)	32 weeks ($n = 11$)
Fatigue	55 ± 21	46 ± 30	44 ± 28	45[a] ± 22	41[a] ± 29
Pain	49 ± 28	31 ± 32	32 ± 29	39 ± 25	34 ± 26
Patient assessment[b]	62 ± 29	38[a] ± 30	49 ± 23	48[a] ± 31	40[a] ± 28
Physician assessment[c]	56 ± 16	30[a] ± 22	36[a] ± 14	31[a] ± 20	26[a] ± 12

Results are given in mean ± standard deviation. [a]Denotes statistical significance of the observed median change-from-baseline value with $P \leq 0.05$ by Wilcoxon signed rank test. [b]Patient self-assessment of overall well-being. [c]Physician global assessment of patient's overall well-being.

Functional assessments

Schirmer-I test

More than half the patients (Figure 2) improved by at least 20% on their lacrimal function through 18 weeks, with 7/15 (47%) still showing improvement at the final visit at 32 weeks. The corresponding improvement rates based on available patients are 73%, 69%, 64%, and 64% at 6, 10, 18, and 32 weeks, respectively.

USF

Improvement by at least 20% in USF was observed in 20%–40% of the patients through 18 weeks (Figure 2), with 7/15 (47%) still showing improvement at the final visit at 32 weeks. The corresponding improvement rates based on available patients are 20%, 46%, 36%, and 64% at 6, 10, 18, and 32 weeks, respectively.

Subjective assessments

Fatigue and patient and physician global assessments

Improvement by at least 20% in fatigue was consistently observed in 40% of the patients through 32 weeks (Figure 2). The corresponding improvement rates based on available patients are 40%, 46%, 43%, and 55% at 6, 10, 18, and 32 weeks, respectively. Complete results on all subjective efficacy measures as assessed by VAS are summarised in Table 2. Statistically significant improvement from baseline was observed in fatigue and in patient and physician global assessments at several time points. There were no notable changes in pain.

Objective assessments of joint counts

Complete results on objective efficacy measures as assessed by joint counts are summarised in Table 3. In general, there were no notable changes except for a statistically significant improvement from baseline in the number of tender joints at 32 weeks.

Laboratory assessments

ESR

Improvement by at least 20% in ESR was observed in 13%–33% of the patients through 18 weeks (Figure 2), with 4/15 (27%) still showing improvement at the final visit of 32 weeks. The corresponding improvement rates based on available patients are 33%, 15%, 14%, and 36% at 6, 10, 18, and 32 weeks, respectively.

CRP

There were no statistically significant changes in CRP at any visit (Table 4).

IgG

No improvement in IgG was observed at 6 weeks, but improvement was seen subsequently in 13%, 7%, and 20% of the patients at 10, 18, and 32 weeks, respectively (Figure 2). The corresponding improvement rates based on available patients are 0%, 15%, 7%, and 27% at 6, 10, 18, and 32 weeks, respectively.

Lymphocytes and Igs

At study entry, peripheral blood lymphocyte and serum Ig levels (mean ± SD) for the 15 patients were as follows: 211 ± 111 B cells per μl, 1034 ± 426 T cells per μl, 1909 ± 669 IgG mg/dl, 291 ± 111 IgA mg/dl, and 146 ± 54 IgM mg/dl. As shown in Table 5 and Figure 3, mean B-cell levels decreased by 54% at 6 weeks, which persisted at subsequent evaluations, with no evidence of onset of recovery by the final evaluation at 32 weeks (6 months post-treatment). In contrast, there were no consistent patterns of decreases/increases either in

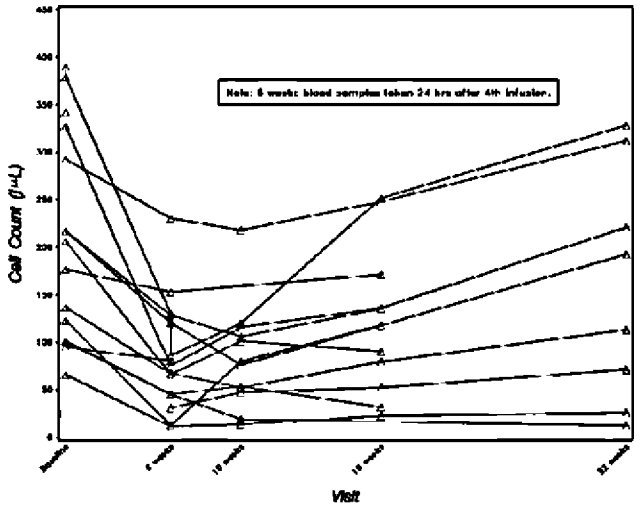
Table 3

Number of tender points and tender joints

Parameters	Baseline ($n = 16$)	6 weeks ($n = 15$)	10 weeks ($n = 13$)	18 weeks ($n = 14$)	32 weeks ($n = 11$)
Tender points	4.1 ± 5.6	2.0 ± 3.1	2.1 ± 3.4	1.6 ± 2.9	2.4 ± 3.3
Tender joints	4.0 ± 7.5	1.0 ± 1.8	1.1 ± 1.6	1.2 ± 1.9	0.3[a] ± 0.5

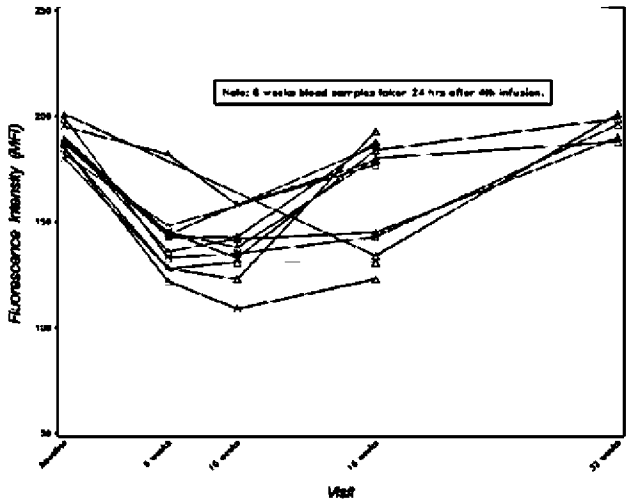
Results are given as mean ± standard deviation. [a]Denotes statistical significance of the observed median change-from-baseline value with $P \leq 0.05$ by Wilcoxon signed rank test. Almost all patients had no swollen joints at baseline or subsequent time points.

Figure 3



Peripheral B-cell counts.

Figure 4



CD22 expression on B cells as measured by mean fluorescence intensity (MFI).

the T-cell levels or in the available serum levels of IgG, IgA, and IgM after treatment.

At study entry, all of the patients with available measurements had a CD22 median fluorescence intensity above the normal range (from median 88 to 201). This is consistent with the finding that patients with Sjögren's syndrome have an over-expression of CD22. Twenty-four hours after treatment with epratuzumab at 6 weeks, all but one patient exhibited a decreased CD22 fluorescence intensity below the normal range (Figure 4). At week 18, five patients remained CD22 downregulated, but the others returned to a fluorescence intensity as high as at entry. At the final evaluation, all the patients recovered to the same increased CD22 expression compared with normal, as observed in patients with untreated pSS.

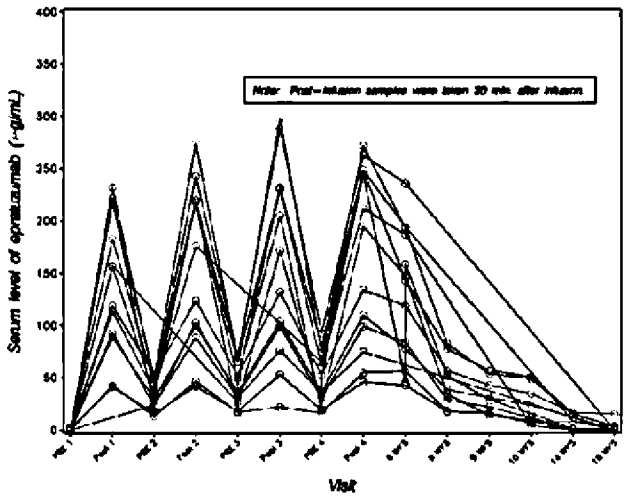
Autoantibodies

There were no changes in the autoantibodies, anti-Ro and anti-La, in any patients with at least one post-treatment measurement; specifically, in subsequent visits, no patient developed new autoantibodies that were not also detected at study entry. Almost all patients (13/15 with at least one post-treatment measurement) had measurable ANA titers (1:80 to 1:10,000) at study entry. At subsequent evaluations, including the 32-week (6 months post-treatment) visit, eight patients exhibited at least twice their baseline ANA titers at one or more evaluations, whereas a similar number (seven patients) had not more than half their baseline titers at one or more evaluations, with two of the aforementioned patients having both increases and decreases from their baseline titers at different evaluation time points.

Study drug administration and reactions

A total of 16 patients were exposed to the study drug. One patient experienced a moderate-severe acute reaction (flushing, dyspnea, nausea, vomiting, nasal mucosa swelling, and glottis pressure) during the first infusion and was discontinued from the study. Of the remaining 15 patients, 14 (93%) completed all four infusions of 360 mg/m^2 of epratuzumab and one prematurely terminated the third infusion after experiencing a moderate grade-3 acute infusion reaction (with a loss of consciousness for several seconds) that subsided within 1 hour (the fourth infusion was not administered to this patient). Overall, the infusions were administered in a median infusion time

Figure 5



Serum levels of epratuzumab, as measured by enzyme-linked immunosorbent assay.

Table 4

Post-treatment changes in CRP, ESR, and Igs

Parameters	Post-treatment change from baseline (mean ± standard deviation)			
	6 weeks	10 weeks	18 weeks	32 weeks
	n = 13	*n* = 13	*n* = 12	*n* = 10
CRP	0.42 ± 1.39	-0.02 ± 0.33	0.19 ± 0.60	0.03 ± 0.22
ESR	1.3 ± 15.0	1.5 ± 11.0	-0.9 ± 7.7	-0.3 ± 11.6
	n = 15	*n* = 13	*n* = 13	*n* = 9
IgG	29.7 ± 249.6	-31 ± 379.6	7.1 ± 313.5	-120.8 ± 363.3
IgA	1.1 ± 24.4	7.8 ± 46.9	-1.2 ± 59	-3.8 ± 62.4
IgM	-14.9 ± 25.3	-9.6 ± 28.3	-15.0 ± 32.8	-2.3 ± 34.8

None of the changes from baseline in the above parameters was statistically significant. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Ig, immunoglobulin.

of 45 minutes (20–150 minutes) and were generally well-tolerated, with four transient AEs (headache, lower limb paresthesia, and two cases of acute infusion reaction) that resolved quickly.

Safety

Safety assessments focus on all 16 patients who were exposed to study medication.

Adverse events

During or after treatment, a total of 10 patients reported AEs. Four reported having a serious AE (drug-related: acute infusion reaction as noted above; non-drug-related: dental abscess, transient ischemic attack with secondary seizure, and osteoporotic fracture), and three patients had a non-serious AE considered drug-related (headache, paresthesia, and acute infusion reaction as noted above) that resolved quickly. The remaining AEs considered unrelated to study medication included fever, palpitation, bone pain, sinusitis, carpal tunnel syndrome, diarrhea, and dyspepsia. The two cases of infection reported above (sinusitis and dental abscess treated with intravenous antibiotics) resolved subsequently without any sequelae.

Safety laboratories

Standard safety laboratories showed no consistent pattern of change from baseline, and infrequent post-treatment increases in National Cancer Institute Common Toxicity Criteria (CTC) (version 3.0) toxicity grades for these laboratories were all limited to changes of at most one grade level, except for one patient with lymphopenia that increased from CTC grade 0 to 2.

Immunogenicity

HAHA analyses showed three patients with elevated values of HAHA: 116 ng/ml at 32 weeks, 120 ng/ml at 18 weeks, and 130 ng/ml at 18 weeks. These isolated cases of low-level positive HAHA are of uncertain clinical significance because they were not associated with specific clinical signs and symptoms or other apparent toxicities.

Pharmacokinetics

Serum samples for analysis of pharmacokinetics by ELISA were collected pre- and post-infusion as well as at 6 weeks (24 hours after fourth infusion) and 8, 9, 10, 14, and 18 weeks. Epratuzumab serum levels were detectable above the 0.5 μg/ml assay limit in all 13 available samples at 6 weeks, in 10/11 samples evaluated at 10 weeks, in 6/10 samples evaluated at 14 weeks, and in 6/14 samples evaluated at 18 weeks, with median values of 143 μg/ml (range, 43–236) at 6 weeks, 14 μg/ml (4–51) at 10 weeks, 11 μg/ml (1–17) at 14 weeks, and 3.9 μg/ml (1–16) at 18 weeks (Figure 5). Non-compartmental pharmacokinetic analysis indicated a serum half-life ($t_{1/2}$) after the fourth infusion of 15 ± 8 days.

Discussion

In this phase I/II open-label study, selective immunomodulation of B cells led to improvement of objective and subjective parameters of disease activity in patients with pSS. In the absence of validated disease activity criteria for pSS, we developed a disease activity score based on the most frequent signs and symptoms of the disease. These included four domains: dryness of the eyes (Schirmer-I test), dryness of the mouth (USF), fatigue (VAS), and laboratory parameters of ESR and/or IgG. Based on this activity score, we observed that more than half (53%) of the patients achieved at least a 20% improvement in at least two domains 24 hours after the fourth infusion at 6 weeks, with the corresponding response rates of

Table 5

Post-treatment changes in lymphocytes

	Post-treatment percentage change from baseline (mean ± SD)			
Parameter	6 weeks	10 weeks	18 weeks	32 weeks
Lymphocytes	$n = 14$	$n = 12$	$n = 11$	$n = 7$
B cells	-54%[a] ± 25%	-45%[a] ± 47%	-39%[a] ± 23%	-31%[a] ± 33%
T cells	2% ± 36%	-6% ± 34%	1% ± 13%	-5% ± 19%

[a]Denotes statistical significance of the observed median change-from-baseline value with $P \leq 0.05$ by Wilcoxon signed rank test. None of the changes from baseline in T cells was statistically significant. SD, standard deviation.

53%, 47%, and 67% at 10, 18, and 32 weeks, respectively. Most improvements occurred in the Schirmer-I test, USF, and fatigue VAS. There appear to be only a few significant changes in ESR or IgG that impacted on the efficacy outcomes. Approximately 40%–50% responded at the improvement level of at least 30%, whereas 10%–45% responded at the 50% improvement level for 10–32 weeks. Interestingly, the number of responders (at 20%, 30%, or 50% improvement levels) was higher 6 months after the treatment administration than earlier. Such results might indicate recovery or regeneration of glandular tissue, suggesting the need for pre- and post-therapy biopsies of minor salivary glands. Additional findings in terms of statistically significant improvement from baseline in fatigue, and in patient and physician global assessments at several time points, also serve to reinforce the positive results based on the composite efficacy endpoint.

B-cell targeting therapies are promising in the treatment of autoimmune diseases, including rheumatoid arthritis [27] and SLE [23]. Also, two studies and some case reports evaluated the anti-CD20 monoclonal antibody, rituximab, in pSS [10-13]. Pijpe *et al.* reported observing a significant improvement in subjective and objective measures, including subjective reports of dryness, fatigue, and salivary flow, mainly in patients with early-onset disease and in only a few with pSS-associated MALT (mucosa-associated lymphoid tissue) lymphoma [11]. Immunologic analysis showed rapid decrease in peripheral B cells but no change in IgG levels. However, four of 14 patients developed human anti-chimeric antibodies (HACAs) and three of them developed a clinical picture compatible with serum sickness [11]. In a retrospective study of short-term efficacy of rituximab in autoimmune diseases, serum-sickness-like diseases occurred in a patient with pSS and in two patients with SLE [12]. This is supported by another French, open-label study using low-dose rituximab which reported improvement in subjective parameters of dryness and which also showed that two of 16 patients developed a clinical picture of serum sickness [13]. The most striking finding in those studies is the observation of HACA-associated serum sickness, which may be of major clinical concern in future trials. Accordingly, fully humanised anti-CD20 monoclonal antibodies are under evaluation in autoimmune diseases, in addition to NHL [28-30]. Nevertheless, all of these CD20 antibodies appear to markedly deplete circulating B cells in treated patients.

Although depleting B cells is interesting in the treatment of autoimmune diseases, a novel and rational approach is modulating their function. Initial data have shown that epratuzumab is effective and safe in the treatment of SLE [23]. This treatment was associated with a modest depletion of B cells (34%–41%) within 18 weeks, as we observed also in the present study (39%–54% within 18 weeks) and as was also found in patients with NHL treated with epratuzumab [22]. However, it might also function by signalling through the inhibitory CD22 molecule, causing down-modulation of BCR signalling, as suggested in recent laboratory studies comparing epratuzumab with rituximab [21]. B-cell homeostasis is disturbed in pSS with diminished frequencies and absolute numbers of peripheral $CD27^+$ memory B cells [4-7]. In addition, we report here, for the first time, that patients with pSS have a CD22 over-expression in their peripheral B cells, which was downregulated by epratuzumab for at least 12 weeks after the therapy.

In addition to assessing any evidence of efficacy, the objective of this open-label phase I/II study was to evaluate the safety of epratuzumab in patients with active pSS. Three patients showed moderately elevated levels of HAHA, but without any specific clinical symptoms or apparent toxicity that could be associated with the elevations. As compared with patients with lymphoma, those suffering from autoimmune diseases have been reported to present a higher rate of antibodies to chimeric rituximab, but usually not related with clinical manifestations [27,31]. These discrepancies may be explained in part by the high B-cell activity in pSS and the lack of concomitant immunosuppressive therapy.

Conclusion

This initial experience in patients with active pSS demonstrated that four doses of 360 mg/m^2 epratuzumab immunotherapy appears to be safe and well-tolerated when infused within 45 minutes, with clinically significant responses observed in approximately half the patients for at least 18 weeks in the presence of modestly decreased (39%–54%)

circulating B-cell levels, and with evidence of minimal immunogenicity, as measured by HAHA. We conclude that epratuzumab may be a promising therapy in patients with active pSS and that a multicentre, randomised, double-blinded, controlled study to confirm the beneficial effects of anti-CD22 therapy is indicated.

Competing interests

SDS and GRB declare research funding for this study provided by Immunomedics, Inc. SDS has acted as a research consultant for Genentech, Inc. NKWT, WAW, and DMG have employment and financial interests (stock) in Immunomedics, Inc., whichowns the antibody tested in this paper. OP and LT declare no competing interests.

Authors' contributions

All authors contributed to data interpretation and the final manuscript. SDS and GRB were the principal investigators and were responsible for all aspects of the study, including patient selection and performing patient-related study procedures. SDS, GRB, DMG, and WAW designed the clinical trial protocol, and NKWT was responsible for data management and statistical analysis. All authors read and approved the final manuscript.

Acknowledgements

The authors acknowledge the patients who agreed to participate in this trial. This study was supported in part by Immunomedics, Inc.

References

1. Bowman SJ, Ibrahim GH, Holmes G, Hamburger J, Ainsworth JR: **Estimating the prevalence among Caucasian women of primary Sjögren's syndrome in two general practices in Birmingham, UK.** *Scand J Rheumatol* 2004, **33**:39-43.
2. Salomonsson S, Jonsson MV, Skarstein K, Brokstad KA, Hjelmstrom P, Wahren-Herlenius M, Jonsson R: **Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjögren's syndrome.** *Arthritis Rheum* 2003, **48**:3187-3201.
3. Stott DI, Hiepe F, Hummel M, Steinhauser G, Berek C: **Antigen-driven clonal proliferation of B cells within the target tissue of an autoimmune disease. The salivary glands of patients with Sjögren's syndrome.** *J Clin Invest* 1998, **102**:938-946.
4. Bohnhorst J, Bjorgan MB, Thoen JE, Natvig JB, Thompson KM: **Bm1–Bm5 classification of peripheral blood B cells reveals circulating germinal center founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjögren's syndrome.** *J Immunol* 2001, **167**:3610-3618.
5. Hansen A, Odendahl M, Reiter K, Jacobi AM, Feist E, Scholze J, Burmester GR, Lipsky PE, Dorner T: **Diminished peripheral blood memory B cells and accumulation of memory B cells in the salivary glands of patients with Sjögren's syndrome.** *Arthritis Rheum* 2002, **46**:2160-2171.
6. Hansen A, Jacobi A, Pruss A, Kaufmann O, Scholze J, Lipsky PE, Dorner T: **Comparison of immunoglobulin heavy chain rearrangements between peripheral and glandular B cells in a patient with primary Sjögren's syndrome.** *Scand J Immunol* 2003, **57**:470-479.
7. Hansen A, Reiter K, Ziprian T, Jacobi A, Hoffmann A, Gosemann M, Scholze J, Lipsky PE, Dorner T: **Dysregulation of chemokine receptor expression and function by B cells of patients with primary Sjögren's syndrome.** *Arthritis Rheum* 2005, **52**:2109-2119.
8. Voulgarelis M, Dafni UG, Isenberg DA, Moutsopoulos HM: **Malignant lymphoma in primary Sjögren's syndrome: a multicenter, retrospective, clinical study by the European Concerted Action on Sjögren's syndrome.** *Arthritis Rheum* 1999, **42**:1765-1772.
9. Tedder T, St Clair E: **New prospects for autoimmune disease therapy: B cells on deathwatch.** *Arthritis Rheum* 2006, **54**:1-9.
10. Somer BG, Tsai DE, Downs L, Weinstein B, Schuster SJ: **Improvement in Sjögren's syndrome following therapy with rituximab for marginal zone lymphoma.** *Arthritis Rheum* 2003, **49**:394-398.
11. Pijpe J, van Imhoff GW, Spijkervet FKL, Roodenburg JLN, Wolbink GJ, Mansour K, Vissink A, Kallenberg CG, Bootsma H: **Rituximab treatment in patients with primary Sjögren's syndrome: an open-label phase II study.** *Arthritis Rheum* 2005, **52**:2740-2750.
12. Gottenberg J, Guillevin L, Lambotte O, Combe B, Allanore Y, Cantagrel A, Larroche C, Soubrier M, Bouillet L, Dougados M, *et al.*: **Tolerance and short-term efficacy or rituximab in 43 patients with systemic autoimmune diseases.** *Ann Rheum Dis* 2005, **64**:913-920.
13. Devauchelle-Pensec V, Morvan J, Pennec Y, Pers JO, Jamin C, Jousse-Joulin S, *et al.*: **Rituximab (anti-CD20) in the treatment of primary Sjögren's syndrome: results of an open label study.** *Ann Rheum Dis* 2005, **64**:S309.
14. Engel P, Nojima Y, Rothstein D, Zhou LJ, Wilson GL, Kehrl JH, Tedder TF: **The same epitope on CD22 of B lymphocytes mediates the adhesion of erythrocytes, T and B lymphocytes, neutrophils and monocytes.** *J Immunol* 1993, **150**:4719-4732.
15. Tedder TF, Poe JC, Haas KM: **CD22: a multifunctional receptor that regulates B lymphocyte survival and signal transduction.** *Adv Immunol* 2005, **88**:1-50.
16. Sato S, Miller AS, Inaoki M, Bock CB, Jansen PJ, Tang MLK, Tedder TF: **CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signalling in CD22-deficient mice.** *Immunity* 1996, **5**:551-562.
17. O'Keefe TL, Williams GT, Batista FD, Neuberger MS: **Deficiency in CD22, a B cell-specific inhibitory receptor, is sufficient to predispose to development of high affinity autoantibodies.** *J Exp Med* 1999, **189**:1307-1313.
18. Carnahan J, Wang P, Kendall R, Chen C, Hu S, Boone T, Juan T, Talvenheimo J, Montestruque S, Sun J, *et al.*: **Epratuzumab, a humanized monoclonal antibody targeting CD22: characterization of *in vitro* properties.** *Clin Cancer Res* 2003, **9**:3982s-3990s.
19. Nitschke L: **The role of CD22 and other inhibitory co-receptors in B-cell activation.** *Curr Opin Immunol* 2005, **17**:290-297.
20. Stein R, Belisle E, Hansen H, Goldenberg DM: **Epitope specificity of the anti-B-cell lymphoma monoclonal antibody, LL2.** *Cancer Immunol Immunother* 1993, **37**:293-298.
21. Carnahan J, Stein R, Qu Z, Hess K, Cesano K, Hansen HJ, Goldenberg DM: **Epratuzumab, a CD22-targeting recombinant humanized antibody with a different mode of action from rituximab.** *Mol Immunol* in press.
22. Leonard JP, Coleman M, Ketas JC, Chadburn A, Ely S, Furman RR, Wegener WA, Hansen HJ, Ziccardi H, Eschenberg M, *et al.*: **Phase 1/II trial of epratuzumab (humanized anti-CD22 antibody) in indolent non-Hodgkin's lymphoma.** *J Clin Oncol* 2003, **21**:3051-3059.
23. Dörner T, Kaufmann J, Wegener WA, Teoh N, Goldenberg DM, Burmester GR: **Initial clinical trial of epratuzumab (humanized anti-CD22 antibody) for immunotherapy of systemic lupus erythematosus.** *Arthritis Res Ther* 2006, **8**:R74.
24. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N, Anderson DR: **Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20.** *Blood* 1994, **83**:435-445.
25. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carson SE, Daniels TE, Fox PC, Fox RI, Kassan SS: **Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group.** *Ann Rheum Dis* 2002, **61**:554-558.
26. Navazesh M: **Methods for collecting saliva.** *Ann N Y Acad Sci* 1993, **694**:72-77.
27. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR, Stevens RM, Shaw T: **Efficacy of B-cell targeted therapy with rituximab in patients with rheumatoid arthritis.** *N Engl J Med* 2004, **350**:2572-2581.
28. Teeling JL, French RR, Cragg MS, van den Brakel J, Pluyter M, Huang H, Chan C, Parren PW, Hack CE, Dechant M, *et al.*: **Char-**

acerization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. *Blood* 2004, 104:1793-1800.

29. Morschhauser F, Leonard JP, Coiffier B, Petillon M, Coleman M, Bahkti A, Sapra P, Teoh N, Wegener WA, Horak ID, *et al.*: **Initial safety and efficacy results of a second-generation humanized anti-CD20 antibody, IMMU-106(hA20), in non-Hodgkin's lymphoma.** *Blood* 2005, **106**:Abstract 2428.
30. McLaughlin P, Grillo-Lopez AJ, Link BK, Levy R, Czuczman MS, Williams ME, Heyman MR, Bence-Bruckler I, White CA, Cabanillas F, *et al.*: **Rituximab chimeric anti-Cd20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program.** *J Clin Oncol* 1998, **16**:2825-2833.
31. Stein R, Qu Z, Chen S, Rosario A, Shi V, Hayes M, Horak ID, Hansen HJ, Goldenberg DM: **Characterization of a new humanized anti-CD20 monoclonal antibody, IMMU-106, and its use in combination with the humanized anti-CD22 antibody, epratuzumab, for the therapy of non-Hodgkin's lymphoma.** *Clin Cancer Res* 2004, 10:2868-2878.

Genentech
IN BUSINESS FOR LIFE

Friday, Mar 17, 2006

Genentech Provides Updates at Annual Investment Meeting

South San Francisco, Calif. -- March 17, 2006 -- Genentech, Inc. (NYSE: DNA) today provided an overview of its business goals for 2006 and beyond, including an update of its Horizon 2010 strategic plan, at its investment community meeting in New York. In addition, the company provided investors with an overview of recent developments, including highlights from its research, development, commercial, and manufacturing efforts.

"Our success over the past several years has transformed our business, leading to additional opportunities for growth," said Chairman and Chief Executive Officer Arthur D. Levinson, Ph. D. "We remain focused on understanding the basic scientific mechanisms of disease, so that we are better able to select the right targets quickly, develop those targets in the clinic, and deliver novel therapies that could change the course of some of the deadliest diseases."

Horizon 2010 Goals
The company provided an update of its long-term business objectives. The company's revised Horizon 2010 goals include:

- To bring at least 20 new molecules into clinical development.
- To bring at least 15 major new products or indications onto the market.
- To become the number one U.S. oncology company in sales.
- To achieve an average compound annual non-GAAP earnings per share[1] growth rate of 25 percent.
- To achieve cumulative free cash flow[2] of $12 billion.

Other Business Updates
The company made the following additional announcements today:

- The company expects approximately 40 to 50 percent growth in non-GAAP earnings per share for the full year 2006.[3]
- The company announced that the anti-CD20 humanized monoclonal antibody (ocrelizumab) Phase I/II ACTION study in rheumatoid arthritis met its primary endpoint of safety in all doses studied and also met its secondary endpoint of clinical activity at all dose levels studied. The most common side effects in ocrelizumab-treated patients included mild to moderate adverse events of nausea, chills or headache around the first infusion. The serious adverse events observed in patients treated with ocrelizumab were similar to those seen in patients receiving placebo. There were no serious infusion related events in patients treated with ocrelizumab. The rates of infection-related adverse events were similar

between placebo and active groups. Further analyses of the data are ongoing and will be submitted for presentation at a future medical meeting.

- The company announced its decision to acquire land in Hillsboro, Oregon for the construction and development of a biotherapeutic fill/finish manufacturing facility, which is expected to be licensed and operational in 2010.
- In February 2006, Genentech purchased from Biogen Idec the NICO clinical manufacturing facility in Oceanside, California, which will add approximately 5,500 liters of capacity to be used for clinical manufacturing of new molecular entities.
- Genentech also announced that the U.S. Food and Drug Administration approved in January 2006 the production of Xolair® (Omalizumab) bulk drug substance at Novartis' production facility in Huningue, France.

Webcast

Genentech will be offering an archived webcast of the investment community meeting on its website at http://www.gene.com. The webcast will be archived and available for replay until 8:00 p.m. Eastern Time on March 31, 2006.

About Genentech

Genentech is a leading biotechnology company that discovers, develops, manufactures and commercializes biotherapeutics for significant unmet medical needs. A considerable number of the currently approved biotechnology products originated from or are based on Genentech science. Genentech manufactures and commercializes multiple biotechnology products and licenses several additional products to other companies. The company has headquarters in South San Francisco, California and is listed on the New York Stock Exchange under the symbol DNA. For additional information about the company, please visit http://www.gene.com.

Notes

[1] Non-GAAP earnings per share estimates for this period exclude the after-tax effects of recurring charges related to the 1999 Roche redemption of our common stock, ongoing special charges related to the City of Hope litigation, stock compensation expense,and any potential special charges related to existing or future litigation or its resolution, or changes in accounting principles, all of which could be significant. [2] Free cash flow, a non-GAAP measure, will be computed by Genentech based on operating cash flow less gross capital expenditures. Operating cash flow is derived from the "net cash provided by operating activities" line in the cash flow statement and excludes the after-tax effects of non-operational items related to our investment portfolio, asset dispositions, litigation costs, debt service costs, and any other potential non-operational items which could affect this line, any of which could be significant. [3] Genentech's forecasted 2006 non-GAAP earnings per share exclude the after tax-effects of recurring charges related to the 1999 Roche redemption of our

common stock estimated to be $105 million on a pretax basis in 2006, special charges related to the City of Hope litigation estimated to be $54 million on a pretax basis in 2006,stock compensation expense associated with Genentech's adoption of SFAS No. 123R on January 1,2006,expected to be in the range of $0.15 to $0.17 per share for 2006 and any other potential special items related to existing or future litigation or its resolution, or changes in accounting principles, all of which could be significant.

This press release contains forward-looking statements regarding growth in non-GAAP earnings per share and cumulative free cash flow; adding 20 new molecules into clinical development and 15 major new products or indications onto the market by 2010; becoming the number one U.S. oncology company in sales; licensure, development and operation of manufacturing facilities; and charges related to the 1999 Roche redemption of Genentech's stock, the City of Hope litigation and stock compensation. Such statements are predictions and involve risks and uncertainties and actual results may differ materially. Among other things, adding molecules into clinical development, adding products or indications into the market, and the licensure, development and operation of manufacturing facilities could be affected by a number of factors, including unexpected safety, efficacy or manufacturing issues,additional time requirements for data analysis, and FDA actions or delays including failure to obtain FDA approval; becoming a leader in oncology sales could be affected by all of the foregoing and by a number of other factors, including competition,pricing, reimbursement,the ability to supply product, product withdrawals and new product approvals and launches; charges related to the 1999 Roche redemption of Genentech's stock, the City of Hope litigation and stock compensation could be affected by a number of factors, including a re-valuation of certain intangible assets, greater than expected litigation-related costs, the number of options granted to employees, Genentech's stock price and certain valuation assumptions concerning Genentech stock; and growth in non-GAAP EPS and cumulative free cash flow, could be affected by all of the foregoing and by a number of other factors, including achieving sales revenue consistent with internal forecasts, unanticipated expenses such as litigation or legal settlement expenses or equity securities writedowns, costs of sales, R&D expenses, fluctuations in contract revenues and royalties, and fluctuations in tax and interest rates. Please also refer to Genentech's periodic reports filed with the Securities and Exchange Commission. Genentech disclaims, and does not undertake, any obligation to update or revise any forward-looking statements in this press release.

[CANCER RESEARCH 58, 3051–3058, July 15, 1998]

Evaluating Antibodies for Their Capacity to Induce Cell-mediated Lysis of Malignant B Cells[1]

Dieter Würflein, Michael Dechant, Bernhard Stockmeyer, Allson L. Tutt, Peisheng Hu, Roland Repp, Joachim R. Kalden, Jan G. J. van de Winkel, Alan L. Epstein, Thomas Valerius,[2] Martin Glennie, and Martin Gramatzki

Division of Hematology/Oncology, Department of Medicine III, University of Erlangen-Nürnberg, 91054 Erlangen, Germany [D. W., M. D., B. S., R. R., J. R. K., T. V., M. Gr.]; Department of Pathology, University of Southern California, Los Angeles, California 90033-4526 [P. H., A. L. E.]; Medarex Europe and Department of Immunology, University Hospital, 3508 GA Utrecht, the Netherlands [J. G. J. v. d. W.]; and Tenovus Research Laboratory, University of Southampton, Southampton SO16 6YD England [A. L. T., M. Gl.]

ABSTRACT

Promising results from clinical trials have led to renewed interest in effector mechanisms operating in antibody-based therapy of leukemia and lymphoma. We tested a panel of B-cell antibodies from the Sixth Human Leukocyte Differentiation Antigen workshop for their capacity to mediate antibody-dependent cellular cytotoxicity, often considered to be one of the most potent effector mechanisms *in vivo*. As effector cells, mononuclear cells and polymorphonuclear (PMN) cells from healthy donors were compared with FcγRI (CD64)-expressing PMN cells from patients receiving granulocyte colony-stimulating factor (G-CSF) treatment. Of the 29 IgG workshop antibodies binding most strongly to the tested malignant human B-cell lines, only 3 consistently induced target cell lysis. These three antibodies were determined to be HLA DR reactive. Experiments with a panel of HLA class II antibodies showed the involvement of individual Fcγ receptors on effector cells to be strongly dependent on the antibody isotype. We then compared killing mediated by chimeric IgG1 antibodies with that from FcγRI-directed bispecific antibodies, targeting classical HLA class II, or the Lym-1 and Lym-2 antigens. The latter two are variant forms of HLA class II, which are highly expressed on the surface of malignant B cells but which are found only at low levels in normal cells. With blood from G-CSF-treated donors, bispecific antibodies showed enhanced killing compared to their chimeric IgG1 derivatives, because they were more effective in recruiting FcγRI-expressing PMN cells. G-CSF- and FcγRI-directed bispecific antibodies to HLA class II, therefore, seem to be an attractive combination for lymphoma therapy.

INTRODUCTION

Malignant lymphomas are the most common neoplasm of young adults, with increasing mortality over the last decades (1). In the Western world, most cases are of B-cell origin, and, although chemo- and radiotherapy have proven to be effective treatments, the majority of patients with disseminated low-grade lymphoma or relapses of high-grade lymphoma will ultimately die from their disease. The application of MoAbs[3] has the potential to become another therapeutic option (2). Hematological malignancies seem to be particularly promising targets for antibody therapy, because antibodies to well-defined and rather specific surface molecules are available, therapeutic antibodies usually reach their targets, and induction of human antimouse or antichimeric antibody is less pronounced than in patients with solid tumors (3). Clinical trials with customized antibodies to patients' tumor idiotype were the first to show encouraging results in lymphoma patients (4), and IDEC-C2B8 [a chimeric CD20 antibody (5)] was the first MoAb to be approved by the United States Food and Drug Administration for treatment in oncology.

MoAbs mediate their antitumor effects either by directly acting on tumor cells (*e.g.*, by blocking growth factors, inhibiting cell proliferation, or inducing programmed cell death or dormancy) or by recruiting immune effector mechanisms such as cell- or complement-dependent cytotoxicity. Studies with isotype switch variants showed a positive correlation between the capacity to induce ADCC *in vitro* and therapeutic efficacy *in vivo*, suggesting that ADCC can be an important mechanism of antibody action *in vivo* (6). Neutrophils, the most abundant Fc receptor-expressing effector cells, showed cytolytic activity against a broad spectrum of tumor cells *in vitro* (7) and were critically involved in the rejection of cytokine-transfected tumor cells *in vivo* (8). *In vitro*, we found cell-mediated target cell lysis by neutrophils to be a major effector mechanism for HER-2/neu-directed MoAbs (9). The contribution of neutrophils can be further enhanced by clinical application of hematopoietic growth factors, such as G-CSF or granulocyte-macrophage CSF, which dramatically raise neutrophil numbers *in vivo* and at the same time stimulate important functions, such as phagocytosis, release of oxygen radicals, and ADCC (10).

Cell-mediated effects of MoAbs require interaction between the Fc region of antibodies with activating Fc receptors on immune effector cells (11). Depending on their specificity for the heavy chains of IgA, IgE, or IgG, Fc receptors are grouped as Fcα, Fcε, or Fcγ receptors, respectively (12, 13). The majority of Fc receptors consist of ligand-specific α chains, which associate with shared molecules for signaling (14, 15). Neutrophils constitutively express the myeloid receptor for IgA (FcαRI, CD89) and two low-affinity IgG receptors, FcγIIa (CD32) and FcγIIIb (CD16; Ref. 16). IFN-γ (17) or G-CSF (18) induces neutrophils to additionally express the high-affinity IgG receptor (FcγRI, CD64). Tumor-cytolytic activity on neutrophils has been established for FcγRI, FcγRII and, more recently, for FcαRI (19) but not for FcγRIIIb, which is glycosylphosphatidylinositol-linked on PMN cells. Monocytes/macrophages mediate tumor cell killing via molecules belonging to all three Fcγ receptor classes, whereas NK cells express only the cytotoxically active FcγRIIIa (11).

In a previous study, comparing the capacity of B cell-directed antibodies to induce ADCC of malignant cells, we observed an unexpected antigen restriction whereby PMN cells induced high levels of target cell lysis with antibodies to HLA class II but not with antibodies to classical B-cell antigens, such as CD19, CD20, CD21, CD37, or CD38 (20). Here, we report on our results with an extended panel of B-cell antibodies from the Sixth Human Leukocyte Differentiation Antigen workshop and with engineered antibody derivatives. The results extend our understanding of HLA class II-directed reagents and their ability to recruit effector cells and underscore the potential of bispecific antibodies as therapeutic reagents in the treatment of lymphoma.

Received 2/11/98; accepted 4/30/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant Va 124/1-3 from the Deutsche Forschungsgemeinschaft.

[2] To whom requests for reprints should be addressed, at Division of Hematology/Oncology, Department of Medicine III, University Erlangen-Nürnberg, Krankenhausstraße 12, 91054 Erlangen, Germany. Phone: 49-9131-853434; Fax: 49-9131-854770.

[3] The abbreviations used are: MoAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; CSF, colony-stimulating factor; G-CSF, granulocyte CSF; PMN, polymorphonuclear; MNC, mononuclear cell; *o*-PDM, *o*-phenlendimaleimide; RFI, relative fluorescence intensity; NK, natural killer.

PATIENTS AND METHODS

Blood Donors. Experiments reported here were approved by the Ethical Committee of the University of Erlangen-Nürnberg, in accordance with the Declaration of Helsinki. After informed consent was obtained, peripheral blood was drawn from healthy volunteers or from patients receiving G-CSF therapy. Patients were treated with rh-met-G-CSF (Neupogen; 3–5 μg/kg of body weight) from Hoffmann La-Roche (Basel, Switzerland), based on clinical indications. In G-CSF treated patients, FcγRI expression on PMN cells was significantly ($P < 0.001$) higher than in healthy donors, as reported (18).

Isolation of Mononuclear and Neutrophil Effector Cells. Mononuclear and neutrophil effector cells were isolated as described (20). Briefly, 10–20 ml of citrate anticoagulated blood was layered over a discontinuous Percoll (Seromed, Berlin, Germany) gradient. After centrifugation, neutrophils were collected at the interphase between the two Percoll layers, and MNCs were collected from the Percoll/plasma interphase. Remaining erythrocytes were removed by hypotonic lysis. Purity of neutrophils was determined by cytospin preparations and exceeded 95%, with few contaminating eosinophils and <1% MNCs. Viability was tested by trypan blue exclusion and was higher than 95%.

Cell Lines. Malignant human B-cell lines REH (O-acute lymphoblastic leukemia), RAJI (Burkitt's lymphoma), ARH-77 (mature B cells), HUT-78 (T-acute lymphoblastic leukemia) and L cells (mouse fibroblasts) were obtained from the American Type Culture Collection (Manassas, VA). RM-1 (an EBV-transformed B-cell line) was from Dr. G. Bonnard (Bethesda, MD; Ref. 21), and JK-6 (plasmocytoma) was established at our institution by Dr. R. Burger (22). All cells were kept in RF10$^+$ medium consisting of RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, 100 units/ml streptomycin, and 4 mmol/liter L-glutamine (all from Life Technologies).

Generation of HLA Class II Transfectants. Vectors containing DRA *0101 (DRα 120; ATCC 57392) and DRB1 *0101 (45.1 DRβ 008; ATCC 57081) were obtained from the American Type Culture Collection. Sense primers CCC-AAG-CTT-CGA-GCT-CTA-CTG-ACT (DRα) and CCC-AAG-CTT-GGT-CCT-GTC-CTG-TTC (DRβ), and antisense primers CCC-TCT-AGA-AAG-TTT-CTT-CAG-TGA (DRα) and CCC-TCT-AGA-GAA-GGT-TCT-TCC-TTG (DRβ) were used to amplify DRα and DRβ, respectively, cDNA from the plasmids and to generate singular *Hin*dIII and *Xba*I restriction sites at 5′ and 3′ ends. PCR products were cloned into pGEM-T-vector (Promega, Madison, WI) and sequenced by the dye terminator method on an ABI Prism automatic sequencer (Applied Biosystems, Foster City, CA). For eukaryotic expression, inserts were ligated via their unique restriction sites into pRc/CMV (Invitrogen, NV Leek, the Netherlands). Transfection of 10^7 L-cells was carried out by simultaneous electroporation of 10 μg of each of the DRα and DRβ expression vectors at 250 V and 960 μF (Bio-Rad, Richmond, CA). After 24 h, G418 (Life Technologies) was added to the culture medium for selection of stable transfectants. High-expressing cells were obtained by fluorescence activated cell sorting on an EPICS ELITE ESP (Coulter, Hialeah, FL) after staining with HLA class II antibody F3.3 and FITC-labeled goat antimouse F(ab′)$_2$.

MoAbs and Antibody Constructs. B-cell panel antibodies were from the Sixth Human Leukocyte Differentiation Antigen workshop (Table 1). Hybridomas of negative control antibody 3.6.2 (mIgG2a) and L243 (HLA DR, mIgG2a) were from the American Type Culture Collection. HLA class II antibody F3.3 (mIgG1) and invariant chain (Ii, CD74) antibody AT14/15 (mIgG1) were produced at Tenovus Research Laboratory (University of Southampton, Southampton, United Kingdom). HLA DR (B8.11.2, mIgG2b)-, DP (B7.21, mIgG2a)-, and DQ (SPV L3, mIgG2a)-specific antibodies were kindly provided by Dr. R. Bontrop (Biomedical Primate Research Center, Rijswijk, the Netherlands). Murine Lym-1 (mIgG2a) and Lym-2 (mIgG1) and their mouse/human chimeric IgG1 constructs were produced as described (23, 24).

Fc receptor antibodies 22 and 197 (mIgG1 and IgG2a, respectively), both to FcγRI; IV.3 (mIgG2b) to FcγRII; and 3G8 (mIgG1) to FcγIII, as well as F(ab′) and F(ab′)$_2$ fragments of IV.3 and 3G8, respectively, were kindly provided by Medarex (Annandale, NJ).

Bispecific antibodies [FcγRI × HLA class II], [FcγRI × Lym-1], and [FcγRI × Lym-2] were produced by chemically cross-linking F(ab′) fragments of trigger molecule MoAb 22 (FcγRI; CD64) with F(ab′) fragments of target antigen antibodies F3.3, Lym-1, or Lym-2, as described (25). Briefly, F(ab′)$_2$ fragments were produced by limited digestion with pepsin and were then reduced with mercaptoethanol amine to provide F(ab′) with free hinge-region sulfur hydroxyl (SH) groups. The SH groups on one of the Fab′γ (SH) partners were then fully derivatized with excess *o*-PDM to provide free maleimide groups. Finally, the F(ab′)-*o*-PDM and F(ab′)-SH were combined at a ratio of 1:1 to generate heterodimeric F(ab′)-*o*-PDM-F(ab′) constructs. After purification by size exclusion chromatography and characterization by high-performance liquid chromatography, samples were sterilized by filtration and stored at 4°C. All bispecific antibodies showed binding to effector and target cells as expected from their parental antibodies.

Chimeric Fab(Fc)$_2$ constructs of HLA class II antibody F3.3, consisting of F(ab′) fragments from the mouse antibody chemically conjugated to two human Fc fragments, were prepared as reported (26). Briefly, F(ab′)-*o*-PDMs of F3.3 were produced as described above. To prepare Fcγ, normal human IgG was digested with papain, and the resulting Fcγ fragments were separated and purified. Following reduction of Fcγ fragments, fragments were incubated with F(ab′)-*o*-PDM to yield Fab(Fc)$_2$ constructs with mainly human IgG1 Fc fragments.

Immunofluorescence Analysis. During incubation of effector cells with MoAbs, polyclonal human IgG (4 mg/ml) was added to inhibit nonspecific binding to FcγRI. FITC-labeled F(ab′)$_2$ fragments of goat antimouse or anti-human MoAbs were used for staining. Cells were analyzed on an EPICS PROFILE flow cytometer (Coulter). For each cell population, RFI was calculated as the ratio of mean linear fluorescence intensity of relevant to irrelevant, isotype-matched antibodies.

ADCC Assays. ADCC assays were performed as described (20). Briefly, target cells were labeled with 200 μCi of ^{51}Cr for 2 h. Effector cells, sensitizing antibodies, and RF10$^+$ were added into round-bottomed microtiter plates. In some experiments, Fcγ receptor-blocking antibodies were used at a final concentration of 10 μg/ml. Assays were started by adding the target cell suspension, giving an E:T cell ratio of 40:1. For whole blood assays, 50 μl of whole blood were added instead of isolated effector cells. After 3 h at 37°C, assays were stopped by centrifugation, and ^{51}Cr release from triplicates was measured in cpm. The percentage of cellular cytotoxicity was calculated using the formula:

Table 1 *Binding of B-cell panel antibodies to B-cell lines of different maturation levels*

One hundred two antibodies from the Sixth Human Leukocyte Differentiation Antigen workshop were tested for binding to four different human B-cell lines. Relative fluorescence intensities, antibody isotypes, and antibody specificity, as determined by the workshop, are listed for the 29 most strongly binding antibodies.

Antibody	Isotype	Antigen	Cell line			
			REH	RAJI	RM-1	JK-6
B001	IgG1	CD45RA	13.9	45.6	44.2	3.4
B005	IgG1	CD138	0.9	0.83	21.4	51.0
B006	IgG1	Unknown	0.9	1.3	29.8	10.7
B019	IgG1	Unknown	13.0	47.7	56.8	1.2
B022	IgG1	CD19	56.6	18.0	4.0	0.9
B023	IgG1	CD22	6.1	21.7	2.0	1.0
B025	IgG1	CD37	1.6	37.0	15.9	0.9
B026	IgG2a	Unknown	1.3	1.7	2.2	19.0
B028	IgG1	CD24	37.7	2.7	2.5	1.2
B033	IgG1	Unknown	1.0	10.3	6.7	4.6
B035	IgG2a	CD55	1.6	1.6	3.4	6.4
B042	IgG1	Unknown	3.0	2.0	23.7	29.3
B044	IgG1	Unknown	11.6	42.1	1.1	50.3
B046	IgG1	CD9	62.4	1.0	0.7	1.3
B048	IgG1	CD79	1.6	7.5	2.1	4.9
B049	IgG1	Unknown	1.1	1.0	1.2	8.3
B054	IgG1	Unknown	9.3	1.3	5.4	1.7
B065	IgG1	Class II	2.0	1.9	30.0	2.6
B066	IgG3	κ	2.0	1.6	0.8	8.0
B067	IgG1	CD39	2.3	2.6	28.4	1.1
B068	IgG2a	Class II	3.0	2.6	12.9	0.5
B069	IgG3	DR	35.8	87.3	67.9	1.9
B070	IgG3	DR	37.2	87.3	56.8	1.1
B071	IgG3	DR	28.9	13.0	6.5	0.8
B076	IgG2a	IgM	2.3	44.2	7.6	0.8
B077	IgG1	CD19	51.3	32.0	6.3	0.6
B095	IgG1	CD21	4.6	25.8	2.1	1.5
B096	IgG1	CD10	52.1	19.2	2.5	1.3
B099	IgG1	CD21	1.4	20.8	1.1	0.8

$$\% \text{ specific lysis} = \frac{\text{Experimental cpm} - \text{basal cpm}}{\text{Maximal cpm} - \text{basal cpm}} \times 100$$

with maximal ^{51}Cr release determined by adding perchloric acid (3% final concentration) to target cells and basal release measured in the absence of sensitizing antibodies and effector cells. Only very low levels of antibody-mediated, noncellular cytotoxicity (without effector cells) were observed under these assay conditions (<5% specific lysis). Antibody independent killing was seen in whole blood assays and with mononuclear effector cells, but not with PMN cells. For analysis of effects induced by Fc receptor antibodies, percentage of inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\% \text{ lysis without} - \% \text{ lysis with Fc}\gamma\text{R antibody}}{\% \text{ lysis without Fc}\gamma\text{R antibody}} \times 100$$

Statistical Analysis. Data are reported as mean ± SE from an indicated number of experiments with different blood donors. Differences between groups were analyzed by unpaired (or, when appropriate, paired) Student's *t* test. Levels of significance are indicated.

RESULTS

ADCC Activity of the B-Cell Panel Antibodies from the Sixth Human Leukocyte Differentiation Antigen Workshop. A total of 102 B-cell antibodies from the Sixth Human Leukocyte Differentiation Antigen workshop were tested for binding to four human B-cell lines representing different stages of maturation: REH (O-acute lymphoblastic leukemia), RAJI (Burkitt's lymphoma), RM-1 (mature B cells), and JK-6 (plasmocytoma). Binding intensities of the 29 most strongly binding IgG antibodies, their isotypes, and their antigen specificities, as determined by the B-cell workshop, are summarized in Table 1. These antibodies were then analyzed for their capacity to mediate ADCC against respective target cell lines. As effector cells, we compared PMN cells (data not shown) and MNCs from healthy donors with PMN cells from patients during G-CSF treatment (Fig. 1). Antibodies B069, B070, and B071 were found to give the highest levels of killing on the broadest spectrum of target cells. These three antibodies, as well as B065 and B068, were determined by the workshop to be HLA class II reactive. B065 and B068, however, bound only weakly to most of the tested target cells (Table 1) and did not mediate ADCC. MNCs, additionally, mediated ADCC against RAJI cells in the presence of antibodies B025 and B099, which were clustered as CD37 and CD21, respectively. Plasmocytoma cell line JK-6 is HLA class II negative and was not lysed with any of the tested panel antibodies. However, JK-6 cells, coated with the hapten nitroiodophenyl, were killed by PMN cells and MNCs in the presence of nitroiodophenyl-directed antibodies, excluding the possibility that they were resistant to the lytic mechanisms of ADCC (data not shown). Interestingly, PMN cells were cytotoxic only in the presence of HLA class II antibodies, as previously reported (20).

Isotype Dependency of MNC- and PMN Cell-mediated ADCC. Antibody isotype has been reported to be a critical factor for effective ADCC (6). Therefore, we analyzed HLA class II antibodies of different isotypes for their capacity to mediate ADCC with PMN cells and mononuclear effector cells. As expected from the isotype specificities of FcγRIIIa on NK cells (16), MNCs were most effective with antibodies of human IgG1 or murine IgG3 isotypes. PMN cells from healthy donors or from G-CSF-treated patients induced significant target cell killing with antibodies of human IgG1, as well as with all

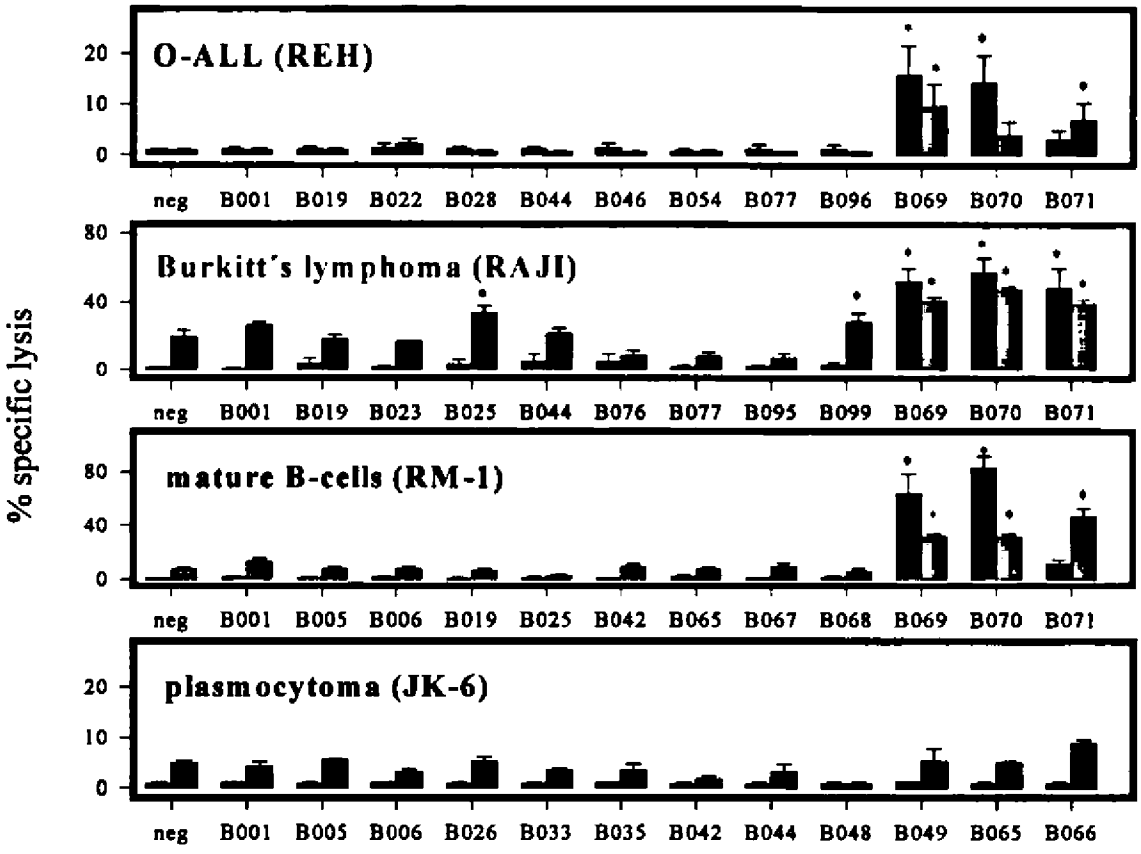


Fig. 1. ADCC capacity of B-cell panel antibodies against malignant B-cell lines. The 12 IgG antibodies with the highest staining intensities on four human B-cell lines (see Table 1) were tested at 2 μg/ml in ADCC against malignant B cells of different maturation levels. As effector cells, G-CSF-primed PMN cells (▩) or MNCs (▨) were used at an E:T ratio of 40:1. Significant ADCC (*) was observed most consistently with antibodies B069, B070, and B071, which were directed against HLA DR and, therefore, stained negative on plasmocytoma cell line JK-6. Results from four experiments with different donors are presented as mean ± SE of the percentage of specific lysis.

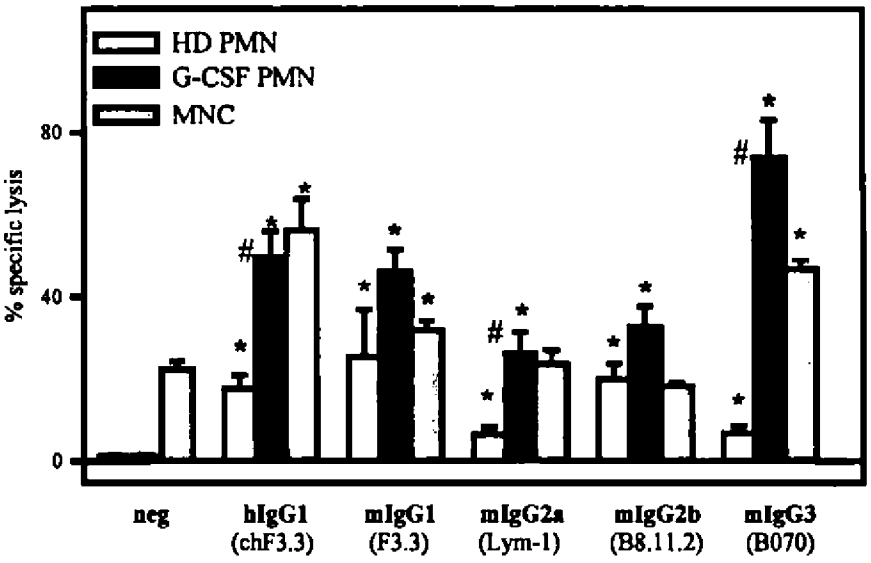


Fig. 2. Isotype dependency of MNC- and PMN cell-mediated ADCC. HLA class II antibodies of different isotypes were compared in ADCC against RAJI lymphoma cells using MNCs, healthy donor PMN cells (*HD PMN*), or G-CSF-primed PMN cells (*G-CSF PMN*) as effector cells. PMN cells mediated significant ADCC ($P < 0.05$, indicated by *) with all antibody isotypes, whereas MNCs were effective only with hIgG1, mIgG3, and mIgG1 isotypes. With antibodies of hIgG1, mIgG2a, and mIgG3 isotypes, G-CSF-primed PMN cells were significantly more cytotoxic ($P < 0.05$, indicated by #) than healthy donor PMN cells. Results from four experiments with different donors are displayed as mean ± SE of the percentage of specific lysis.

murine isotypes (Fig. 2). G-CSF-primed PMN cells were significantly more effective than healthy donor PMN cells with human IgG1, murine IgG2a, and murine IgG3 antibodies. From these data, we conclude that negative results with G-CSF-primed PMN cells and B-cell panel antibodies (Fig. 1) were not explained by insufficient interaction of sensitizing murine antibodies with human Fc receptors on PMN cells.

Isotype-dependent Involvement of FcγR in ADCC by G-CSF-primed PMN Cells. MNC-mediated ADCC was, as expected, completely blocked by $F(ab')_2$ fragments of FcγRIII antibody 3G8 (data not shown). In contrast to healthy donor PMN cells, which express both the low-affinity FcγRII (CD32) and FcγRIII (CD16), G-CSF-primed PMN cells additionally express the high-affinity FcγRI (CD64). The contribution of each of these three FcγR classes to ADCC was analyzed in assays via different target antibody isotypes by selectively blocking FcγRI, FcγRII, or FcγRIII with antibodies 197, IV.3, or 3G8, respectively (Fig. 3). Under our assay conditions, these Fc receptor antibodies are well documented to block selectively their respective Fc receptors (Refs. 13 and 16). Involvement of FcγRI was highest in assays via mIgG3, mIg2a, and hIgG1 antibodies. These three isotypes were also more effective with G-CSF-primed PMN cells compared to healthy donor PMN cells (Fig. 2), indicating that expression of FcγRI on G-CSF-primed PMN cells causes enhanced killing via these isotypes. Blocking of FcγRII, on the other hand, had the most prominent influence in assays via mIgG1 and mIgG2b isotypes (in decreasing order). Interestingly, blockade of the most strongly expressed FcγRIII did not induce significant inhibition in assays via any of these target antibody isotypes, a finding that might relate to the glycosylphosphatidylinositol anchorage of FcγRIIIb in PMN cells. However, $F(ab')_2$ fragments of FcγRIII antibody 3G8 stimulated ADCC in FcγRII-dependent assays. In combination with data from Fig. 2, these results suggest that particularly the therapeutic

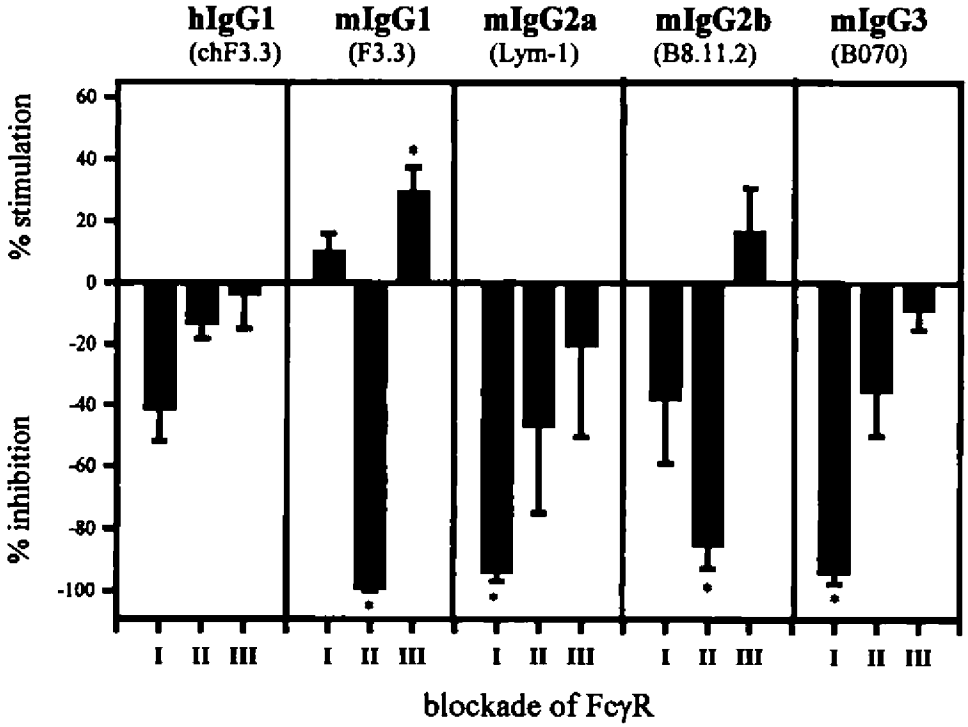


Fig. 3. Role of different FcγR in ADCC by G-CSF-primed PMN cells, depending on target antibody isotypes. G-CSF-primed PMN cells, expressing FcγRI, FcγRII, and FcγRIII, were used as effector cells in ADCC against RAJI lymphoma cells. Target cells were sensitized with HLA class II antibodies of different isotypes, and FcγR involvement was determined by blocking FcγRI (*I*), FcγRII (*II*), or FcγRIII (*III*) with MoAb 197 (whole antibody), IV.3 [F(ab') fragments], or 3G8 [$F(ab')_2$-fragments], respectively. Involvement of FcγRI, as indicated by blockade with MoAb 197, was highest with mIgG3 antibody (94 ± 4%), whereas blockade of FcγRII had the strongest influence on mIgG1-mediated ADCC (98 ± 1%). Blockade of FcγRIII showed no significant inhibition with any of the tested isotypes, but it stimulated FcγRII-mediated ADCC via mIgG1 and mIgG2b antibodies. Data represent mean ± SE of percentage of inhibition or stimulation from four experiments with different donors. *, significant influences of FcγR antibodies.

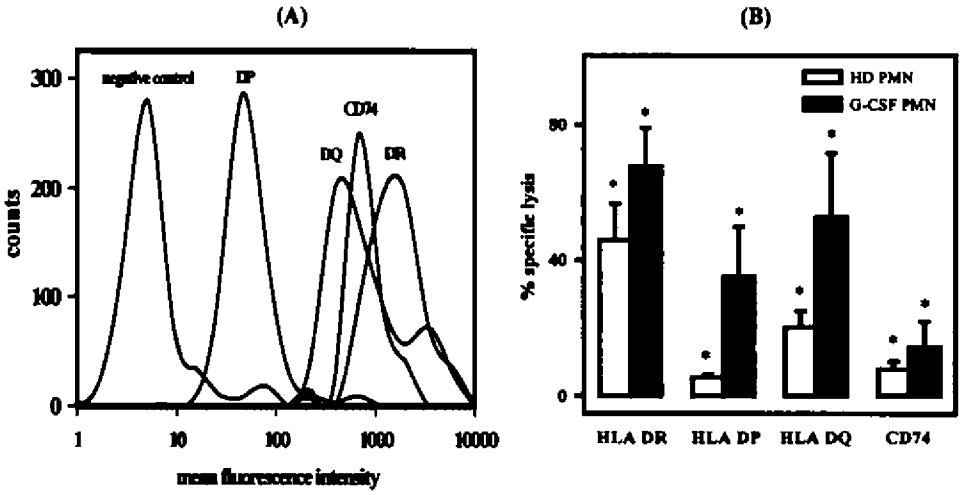


Fig. 4. Comparing HLA DR, DP, DQ and CD74 as target antigens on malignant B cells. *A*, antibodies against HLA DR (B8.11.2), DP (B7/21), DQ (SPV-L3), or CD74 (AT14/19) were used for sensitization of RM-1 mature B cells. *B*, in ADCC assays with PMN cells from healthy donors (*HD PMN*) or from G-CSF-treated patients (*G-CSF PMN*), both effector cell populations mediated significant killing ($P < 0.05$, indicated by *) by antibodies to all four target antigens. G-CSF-primed PMN cells were more effective than healthy donor PMN cells. Data from four experiments with different donors are presented as mean ± SE of the percentage of specific lysis.

efficacy of antibody isotypes reacting with FcγRI may benefit from a combination with G-CSF.

Comparing HLA DR, DP, DQ, and Invariant Chain (Ii, CD74) as Target Antigens. With the B-cell panel antibodies, PMN cells only mediated ADCC against malignant B-cells with HLA DR antibodies but not with antibodies to other B cell-related antigens (Fig. 1). Next, we analyzed whether PMN cells could induce B-cell lysis with antibodies to other HLA class II isoforms, or to the associated invariant chain (Ii, CD74). RM-1 mature B cells served as targets for these experiments because they were found to express high levels of all four antigens (Fig 4*A*). As shown in Fig 4*B*, both healthy donor and G-CSF-primed PMN cells induced significant ADCC with antibodies to HLA DR, DP, DQ, or via invariant chain antibodies. G-CSF-primed PMN cells were again more effective than healthy donor PMN cells.

To analyze whether neutrophil-mediated cytotoxicity by HLA class II antibodies is B cell specific, we used the HLA class II expressing T-cell line HUT-78 (RFI = 207) as the target for ADCC. In these experiments, healthy donor PMN cells were highly effective (40 ± 15% specific lysis; $n = 6$). Next, we tested whether HLA DR transfected into nonlymphoid cells can trigger ADCC in the presence of HLA class II antibodies. For this purpose, HLA DRα and DRβ were stably cotransfected into L cells (RFI = 55), which were then used as targets in ADCC. Again, effective ADCC was mediated by PMN cells in the presence of HLA class II antibody F3.3 (17 ± 7% specific lysis; $n = 4$).

Comparison of FcγRI-directed Bispecific Antibodies and Chimeric Human IgG1 Antibodies against HLA Class II and Its Variants Lym-1 and Lym-2. Results reported thus far were obtained with isolated effector cells at constant effector to target cell ratios. In addition to inducing FcγRI expression on PMN cells, *in vivo* application of G-CSF also dramatically increases PMN cell numbers. To assess the role of increased effector cell numbers, we established whole blood ADCC assays, in which 50 μl of freshly drawn blood from healthy donors or from patients receiving G-CSF were used as the effector sources. These assays, in addition to cell-mediated effects, measured antibody-mediated complement-dependent lysis. FcγRI-directed bispecific antibodies (all [F(ab') × F(ab')]) to classical HLA class II (22 × F3.3]), Lym-1 (22 × Lym-1), or Lym-2 (22 × Lym-2) antigens were compared with their respective mouse/human chimeric IgG1 constructs. ARH-77 mature B cells were used as targets in these assays because they expressed all three target antigens (Fig 5*A*). All three chimeric IgG1 constructs mediated significant lysis with blood from healthy donors (data not shown) or from G-CSF-treated patients (Fig 5*B*), especially at high antibody concentrations. However, there was no significant difference between healthy donor blood and blood from G-CSF-treated donors. Interestingly, whole blood from G-CSF-treated donors mediated ADCC with all three bispecific antibodies, and this was significantly higher than the cytotoxicity achieved with chimeric antibodies, especially with Lym-1- or Lym-2-directed bispecific antibodies (Fig 5*B*). As expected from the low numbers of FcγRI-expressing cells in healthy donors, FcγRI-directed bispecific antibodies were not effective with blood from healthy donors (data not shown).

When whole blood was then fractionated into plasma, MNCs, and PMN cells, the chimeric antibody against classical HLA class II was significantly more effective in inducing lysis with fresh human plasma than the Lym-1 or Lym-2 antibodies. This activity was completely abolished when plasma was heat inactivated, suggesting that complement activation was the underlying mechanism. Chimeric IgG1 antibodies directed against Lym-1 (data not shown) or Lym-2 (Fig. 6) mediated lysis mainly with mononuclear effector cells. However, FcγRI-directed bispecific antibodies also effectively recruited G-CSF-primed PMN cells and therefore were significantly more active with G-CSF-primed blood than chimeric IgG1 antibodies.

DISCUSSION

Results reported in this study extend previous observations that HLA class II antibodies are excellent in eliciting effector cell-mediated killing of malignant B cells (20, 27, 28). Antibodies to HLA class II were also reportedly effective in mediating complement-dependent lysis (29), in inhibiting cell proliferation (30), and in inducing apoptosis by Fas-mediated pathways (31), all of which are considered important effector mechanisms for therapeutic antibodies *in vivo*. HLA class II antigens are highly expressed on a broad spectrum of malignant B cells, are absent on hematopoietic stem cells and plasma cells, do not modulate, and are only found at low levels in soluble form. Furthermore, antibodies to HLA class II showed therapeutic efficacy against malignant B cells in syngeneic (32) and xenografted mouse tumor models (30, 33). Importantly, no side effects were observed in these mice, and extended immunological experiments did not reveal long-lasting immunosuppression after this treatment (32). However, HLA class II is not specific for malignant B cells but is also found on antigen-presenting cells like normal B cells, monocytes/macrophages, and dendritic cells. On these cells, HLA class II serves important functions in presenting antigens to CD4-positive T cells, and HLA class II antibodies were shown to induce tolerance under

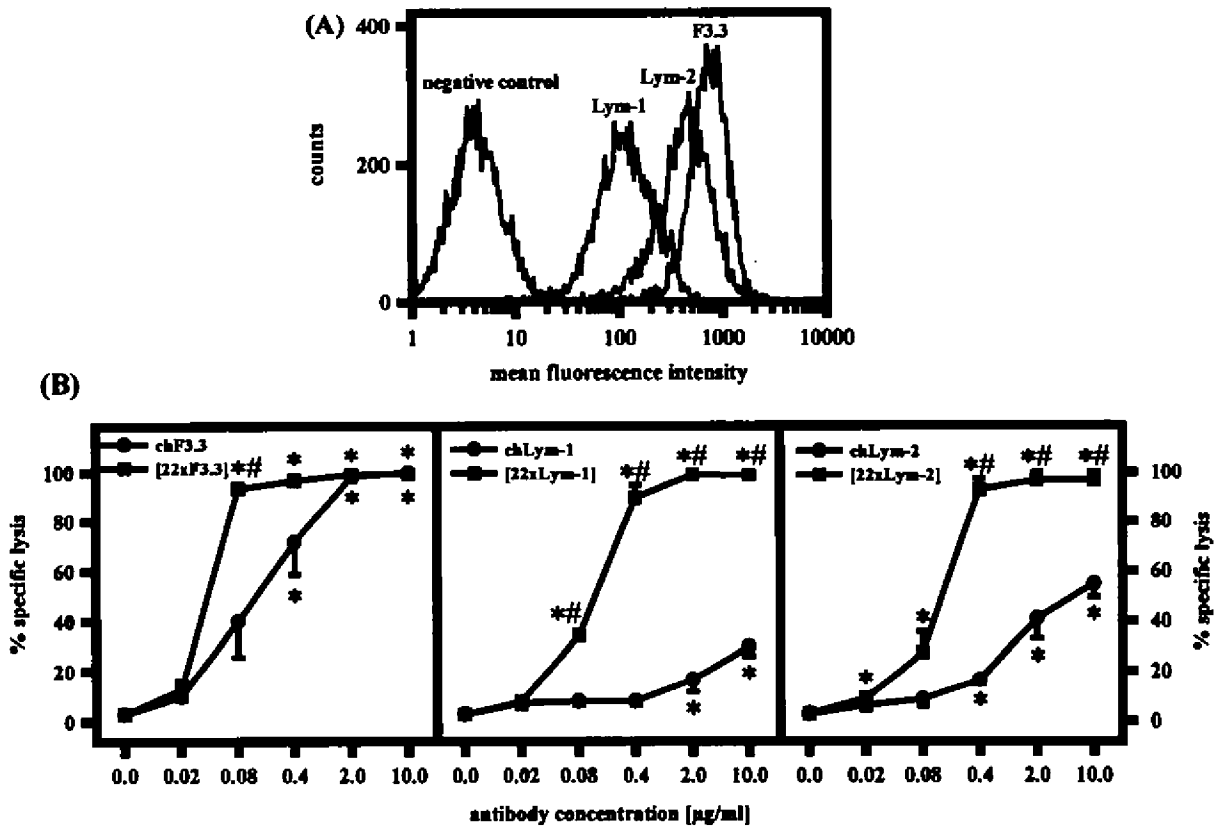


Fig. 5. Comparing chimeric IgG1 and FcγRI-directed bispecific antibodies against "classical" HLA class II and against HLA class II variants Lym-1, or Lym-2. *A*, whole blood from G-CSF-treated patients was used as effector source against ARH-77 mature B cells, which stained positive with HLA class II antibody F3.3, Lym-1, and Lym-2. *B*, significant lysis ($P < 0.05$, indicated by *) was observed with all six constructs. However, bispecific antibodies were significantly more effective ($P < 0.05$, indicated by #) than chimeric IgG1 antibodies, especially when Lym-1 or Lym-2 was targeted. Data from three experiments with different donors are presented as mean ± SE of the percentage of specific lysis.

certain experimental conditions (34). In addition, HLA class II expression is inducible on virtually every cell type by proinflammatory cytokines, such as IFN-γ. Subsequently, HLA class II antibodies showed severe toxicity in nonhuman primates, probably due to uncontrolled complement activation on cytokine-activated endothelial cells (35). These latter results have delayed the clinical development of HLA class II antibodies as therapeutic reagents because constructs with a lower complement-activating capacity were needed.

Reduced complement activation by MoAbs can be achieved by different approaches. For example, the C1Q-binding site of human IgG could be mutated, or antibody isotypes with less activity in complement activation, such as human IgG4 or human IgA, could be selected. Human IgA does not activate complement-dependent lysis, which seems beneficial when HLA class II is considered as target antigen, but is very effective in inducing cell-mediated lysis of tumor cells (19). Furthermore, antibodies to glycosylation variants of HLA class II, such as Lym-1 or Lym-2, have been shown to activate human complement (29) but were less potent than classical HLA class antibodies. Lym-1 and Lym-2 may have the additional advantage that they bind preferentially to HLA class II in malignant human B cells

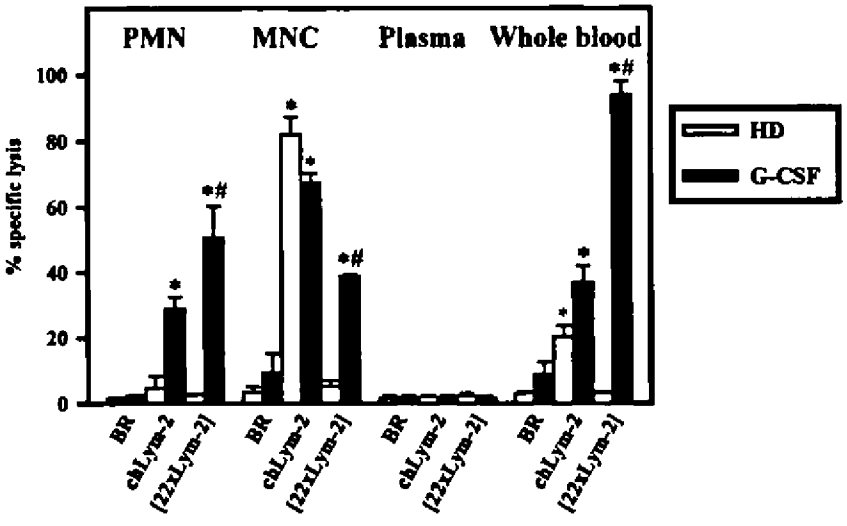


Fig. 6. Analyzing effector mechanisms of a chimeric IgG1 and an FcγRI-directed bispecific antibody against Lym-2. Lysis of ARH-77 mature B cells was measured comparing mouse/human chimeric IgG1 or (FcγRI × Lym-2) bispecific antibody directed to the Lym-2 antigen (both 2 µg/ml). As the effector source, whole blood from G-CSF-treated patients (*G-CSF*) or from healthy donors (*HD*) was compared and then fractionated into plasma, isolated MNCs or PMN cells. Interestingly, plasma was completely ineffective, indicating that no complement-mediated lysis occurred. In healthy donor blood, lysis with the chimeric IgG1 antibody resided mainly in the MNC fraction, whereas in G-CSF-treated patients, PMN cells were also recruited more effectively. As expected, the FcγRI-directed bispecific antibody was only effective with G-CSF-primed samples, in which it induced significantly higher killing ($P < 0.05$) than the chimeric antibody. *, significant lysis; #, differences between healthy donors and G-CSF-treated patients ($P < 0.05$).

compared to normal B cells and monocytes (23). A clinical Phase I trial with murine Lym-1 showed minimal toxicity in lymphoma patients (36). However, clinical responses with the unconjugated antibody were unsatisfactory, and the Lym-1 antibody is currently evaluated as a radioimmunoconjugate (37, 38). Meanwhile, antibodies Lym-1, Lym-2, and 1D10 (another antibody with similar binding characteristics) have been expressed as human IgG1 antibodies (30, 39), and clinical trials with these reagents are expected to start soon. However, in whole blood assays, effector cell recruitment by human IgG1 antibodies proved less optimal, as shown in Fig 5*B*.

Human IgG1 antibodies effectively activate human complement, interact well with FcγRIIIa on NK cells and macrophages (40), and have an extended half-life *in vivo* because they are protected from degradation by binding to FcRB (41). However, human IgG1 was less effective in recruiting PMN cells, the most populous Fc receptor-expressing effector cells in the blood. This could be explained by competition of therapeutic antibodies with high concentrations of natural immunoglobulins for binding to Fc receptors. This issue is especially critical for FcγRI because this high-affinity receptor binds monomeric IgG and therefore is not available as cytotoxic trigger molecule in the presence of serum concentrations of human IgG (42). In addition, therapeutic antibodies may bind to Fc receptors on non-effector cells (*e.g.*, platelets or B cells) or to Fc receptors on effector cells, which do not trigger cytolytic cascades (*e.g.*, FcγRIIb or FcγRIIIb). Fc receptor-directed bispecific antibodies represent an elegant solution to many of these problems (43). These genetically or chemically constructed molecules combine specificity for a tumor cell epitope with reactivity for a cytotoxic trigger molecule on immune effector cells, thereby allowing specific engagement of activating Fc receptors on cytotoxic cells. By selecting antibodies, which bind with their variable regions to Fc receptor epitopes distinct from the immunoglobulin binding site, competition with serum immunoglobulin can be avoided, and full activity in the presence of natural antibodies is conserved. Hematopoetic growth factors, such as G-CSF or granulocyte-macrophage CSF, can be used to dramatically enhance efficacy of Fc receptor-directed bispecific antibodies by increasing effector cell numbers and by up-regulating expression of select Fc receptors (44). Recently, we proposed FcαRI, which is found on monocytes/macrophages as well as eosinophilic and neutrophilic granulocytes, as promising trigger molecule (19). Bispecific antibodies directed against FcγRI (which is expressed on monocytes/macrophages, activated PMN cells, and subpopulations of dendritic cells) and FcγRIII (which is expressed on macrophages and NK cells) are currently being tested, with promising results, in clinical trials (43). In Phase I/II studies, Fc receptor-directed bispecific antibodies showed acceptable toxicity profiles and evidence of biological activity (45–48), leading to an ongoing evaluation for clinical efficacy (49). The results presented in this report provide the rationale for clinical studies with a combination of G-CSF- and FcγRI-directed bispecific antibodies to HLA class II-related antigens in lymphoma patients.

ACKNOWLEDGMENTS

We thank S. Gehr, B. Bock, and T. Schönmetzler for their expert technical assistance. We acknowledge Drs. D. Mason and R. van Lier and their groups for characterizing the antibodies of the B-cell panel from the Sixth Human Leukocyte Differentiation Antigen workshop, and we thank Prof. G. Stevenson for his support producing antibody derivatives.

REFERENCES

1. Bailar, J. C., III, and Gornik, H. L. Cancer undefeated. N. Engl. J. Med., *336:* 1569–1574, 1997.
2. Dillman, R. O. Antibodies as cytotoxic therapy. J. Clin. Oncol., *12:* 1497–1515, 1994.
3. Grossbard, M. L., Press, O. W., Appelbaum, F. R., Bernstein, I. D., and Nadler, L. M. Monoclonal antibody-based therapies of leukemia and lymphoma. Blood, *80:* 863–878, 1992.
4. Miller, R. A., Maloney, D. G., Warnke, R., Levy, R. Treatment of B-cell lymphomas with monoclonal antiidiotype antibody. N. Engl. J. Med., *306:* 517–522, 1982.
5. Reff, M. E., Carner, K., Chambers, K. S., Chinn, P. C., Leonard, J. E., Rabb, R., Newman, R. A., Hanna, N., Anderson, D. R. Depletion of B-cells *in vivo* by a chimeric mouse human monclonal antibody to CD20. Blood, *83:* 435–445, 1994.
6. Dyer, M. J. S., Hale, G., Hayhoe, F. G. J., and Waldman, H. Effects of Campath-1 antibodies *in vivo* in patients with lymphoid malignancies: influence of antibody isotype. Blood, *73:* 1431–1439, 1989.
7. Lichtenstein, A. Granulocytes as possible effectors of tumor immunity. *In:* H. F. Oettgen (ed.), Human Cancer Immunology I, pp. 731–746. Philadelphia: W. B. Saunders, 1990.
8. Musiani, P., Modesti, A., Giovarelli, M., Cavallo, F., Colombo, M. P., Lollini, P. L., and Forni, G. Cytokines, tumour-cell death and immunogenicity: a question of choice. Immunol. Today, *18:* 32–36, 1997.
9. Stockmeyer, B., Valerius, T., Repp, R., Heijnen, I. A. F. M., Bühring, H. J., Deo, Y. M., Kalden, J. R., Gramatzki, M., and van de Winkel, J. G. J. Preclinical studies with FcγR bispecific antibodies and G-CSF primed neutrophils as effector cells against HER-2/neu overexpressing breast cancer. Cancer Res., *57:* 696–701, 1997.
10. Platzer, E. Human hematopoietic growth factors. Eur. J. Haematol., *42:* 1–15, 1989.
11. Fanger, M. W., Shen, L., Graziano, R. F., and Guyre, P. M. Cytotoxicity mediated by human Fc receptors for IgG. Immunol. Today, *10:* 92–99, 1989.
12. Morton, H. C., van Egmond, M., and van de Winkel, J. G. J. Structure and function of human IgA Fc receptors (FcαR). Crit. Rev. Immunol., *16:* 423–440, 1996.
13. Ravetch, J. V. Fc receptors. Curr. Opin. Immunol., *9:* 121–125, 1997.
14. Cambier, J. C. Antigen, and Fc receptor signalling. J. Immunol., *155:* 3281–3285, 1995.
15. Indik, Z. K., Park, J. G., Hunter, S., and Schreiber, A. D. The molecular dissection of Fcγ receptor mediated phagocytosis. Blood, *86:* 4389–4399, 1995.
16. van de Winkel, J. G. J., and Capel, P. J. A. Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. Immunol. Today, *14:* 215–221, 1993.
17. Guyre, P. M., Morganelli, P. M., and Miller, R. Recombinant immune interferon increases immunoglobulin Fc receptors on cultured human mononuclear phagocytes. J. Clin. Invest., *72:* 393–397, 1983.
18. Repp, R., Valerius, T., Sendler, A., Gramatzki, M., Iro, H., Kalden, J. R., and Platzer, E. Neutrophils express the high affinity receptor for IgG (Fc-gamma RI; CD64) after *in vivo* application of rhG-CSF. Blood, *78:* 885–889, 1991.
19. Valerius, T., Stockmeyer, B., van Spriel, A. B., Graziano, R. F., van den Herik-Oudijk, I. E., Repp, R., Deo, Y. M., Lund, J., Kalden, J. R., Gramatzki, M., and van de Winkel, J. G. J. FcαRI (CD89) as a novel trigger molecule for bispecific antibodies. Blood, *90:* 4485–4492, 1997.
20. Elsässer, D., Valerius, T., Repp, R., Weiner, G. J., Deo, Y., Kalden, J. R., van de Winkel, J. G. J., Stevenson, G. T., Glennie, M. J., and Gramatzki, M. HLA class II as potential target antigen on malignant B cells for therapy with bispecific antibodies in combination with G-CSF. Blood, *87:* 3803–3812, 1996.
21. Bonnard, G. D., Yasaka, K., and Maca, R. D. Continued growth of functional human T lymphocytes: production of human T-cell growth factor. Cell. Immunol., *51:* 390–401, 1980.
22. Burger, R., Gebhardt, E., Trautmann, U., Hansen-Hagge, T. E., Strobel, G., Bartram, C., Kalden, J. R., and Gramatzki, M. Two new interleukin-6 dependent plasma cell lines carrying a chromosomal abnormality involving the IL-6 gene locus. Br. J. Haematol., *87:* 212, 1994.
23. Epstein, A. L., Marder, R., Winer, J. N., Stathopoulos, E., Chen, F. M., Parker, J. W., and Tyalor, C. R. Two new monoclonal antibodies, Lym-1 and Lym-2, reactive with human B-lymphocytes and derived tumors, with immunodiagnostic and immunotherapeutic potential. Cancer Res., *47:* 830–840, 1987.
24. Hu, P., Glasky, M. S., Yun, A., Alauddin, M. M., Hornick, J. L., Khawali, L. A., and Epstein, A. L. A human-mouse chimeric Lym-1 monoclonal antibody with specificity for human lymphomas expressed in a baculovirus system. Hum. Antib. Hybrid., *6:* 57–67, 1995.
25. Glennie, M. J., Tutt, A. L., and Greenman, J. Preparation of multispecific $F(ab')_2$ and F(ab')3 antibody derivatives. *In:* G. Gallagher, R. C. Rees, and C. W. Reynolds (eds.), Tumour Immunobiology: A Practical Approach, pp. 225–244. Oxford: Oxford University Press, 1995.
26. Stevenson, G. T., Glennie, M. J., and Kan, K. S. Chemically engineered chimeric and multi-Fab antibodies. *In:* M. Clark (ed.), Protein Engineering of Antibody Molecules, pp. 127–138. Nottingham, United Kingdom: Academic Titles, 1993.
27. Vaickus, L., Biddle, W., Cemerlic, D., and Foon, K. A. Interferon γ augments Lym-1-dependent, granulocyte-mediated tumor cell lysis. Blood, *75:* 2408–2416, 1990.
28. Cemerlic, D., Dadey, B., Han, T., and Vaickus, L. Cytokine influence on killing of fresh chronic lymphocytic leukemia cells by human leukocytes. Blood, *77:* 2707–2715, 1991.
29. Ottonello, L., Morone, P., Dapino, P., and Dallegri, F. Monoclonal Lym-1 antibody-dependent lysis of B-lymphoblastoid tumor targets by human complement and cytokine-exposed mononuclear and neutrophilic polymorphonuclear leukocytes. Blood, *87:* 5171–5178, 1996.
30. Funakoshi, S., Hirano, A., Beckwith, M., Asai, O., Tian, Z., Hornick, J. L., Hu, P., Khawli, L. A., Epstein, A. L., Longo, D. L., and Murphy, W. J. Antitumor effects of nonconjugated murine Lym-2 and human-mouse chimeric CLL-1 monoclonal anti-

bodies against various human lymphoma cell lines *in vitro* and *in vivo*. Blood, *90:* 3160–3166, 1997.
31. Truman, J. P., Choqueux, C., Tschopp, J., Vedrenne, J., Le Deist, F., Charron, D., and Mooney, N. HLA class II-mediated death is induced via Fas/Fas ligand interactions in human splenic B lymphocytes. Blood, *89:* 1996–2007, 1997.
32. Bridges, S. H., Kruisbeek, A. M., and Longo, D. L. Selective *in vivo* antitumor effects of monoclonal anti-I-A antibody on a B cell lymphoma. J. Immunol., *139:* 4242–4249, 1987.
33. Gill, I., Agah, R., Hu, E., and Mazumder, A. Synergistic effects of interleukin 2 and the monoclonal Lym-1 against human Burkitt lymphoma cells *in vitro* and *in vivo*. Cancer Res., *49:* 5377–5379, 1989.
34. Fultz, M., Carman, J., Finkelman, F. D., and Mond, J. J. Neonatal suppression with anti-Ia antibody. III. *In vivo* responses to the type 2 antigen TNP-ficoll. J. Immunol., *143:* 403–406, 1989.
35. Marx, J. L. Suppressing autoimmunity in mice. Science (Washington DC), *221:* 843–845, 1983.
36. Hu, E., Epstein, A. L., Naeve, G. S., Gill, I., Martin, S., Sherrod, A., Nichols, P., Chen, D., Mazumder, A., and Levine, A. M. A Phase Ia clinical trial of Lym-1 monoclonal antibody serotherapy in patients with refractory B cell malignancies. Hematol. Oncol., *7:* 155–166, 1989.
37. DeNardo, G. L., Lewis, J. P., DeNardo, S. J., and O'Grady, L. F. Effect of Lym-1 radioimmunoconjugate on refractory chronic lymphocytic leukemia. Cancer (Phila.), *73:* 1425–1432, 1994.
38. Lewis, J. P., De Nardo, G. L., and DeNardo, S. J. Radioimmunotherapy of lymphoma: a UC Davis experience. Hybridoma, *14:* 115–120, 1995.
39. Gingrich, R. D., Dahle, C. E., Hoskins, K. F., and Senneff, M. J. Identification and characterization of a new surface membrane antigen found predominantly on malignant B lymphocytes. Blood, *75:* 2375–2387, 1990.
40. Brüggemann, M., Williams, G. T., Bindon, C. I., Clark, M. R., Walker, M. R., Jefferis, R., Waldmann, H., and Neuberger, M. S. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. J. Exp. Med., *166:* 1351–1361, 1987.
41. Ghetie, V., and Ward, E. S. FcRn: the MHC class I-related receptor that is more than an IgG transporter. Immunol. Today, *18:* 592–598, 1997.
42. Heijnen, I. A. F. M., Rijks, L. J. M., Schiel, A., Stockmeyer, B., van Ojik, H. H., Dechant, M., Valerius, T., Keler, T., Tutt, A. L., Glennie, M. J., van Royen, E. A., Capel, P. J. A., and van de Winkel, J. G. J. Generation of HER-2/neu-specific cytotoxic neutrophils *in vivo*: efficient arming of PMN by combined administration of G-CSF and FcγRI bispecific antibodies. J. Immuonol., *159:* 5629–5639, 1997.
43. Deo, Y. M., Graziano, R. F., Repp, R., and van de Winkel, J. G. J. Clinical significance of IgG receptors and FcγR-directed immunotherapies. Immunol. Today, *18:* 127–135, 1997.
44. Valerius, T., Stockmeyer, B., and van de Winkel, J. G. J. Use of Filgrastim (r-metHuG-CSF) to enhance Fc receptor function. *In:* G. Morstyn and T. M. Dexter (eds.), Filgrastim (r-metHuG-CSF) in Cinical Practice, Ed. 2, pp. 73–92. New York: Marcel Dekker, 1998.
45. Valone, F. H., Kaufman, P. A., Guyre, P. M., Lewis, L. D., Memoli, V., Deo, Y., Graziano, R., Fisher, J. L., Meyer, L., Mrozek-Orlowski, M., Wardwell, K., Guyre, V., Morley, T. L., Azvizu, C., and Fanger, M. W. Phase Ia/Ib trial of bispecific antibody MDX-210 (anti-Her-2/neu × anti-FcγRI) in patients with advanced breast or ovarian cancer that overexpresses the proto-oncogene HER-2/neu. J. Clin. Oncol., *13:* 2281–2292, 1995.
46. Curnow, R. T. Clinical experience with CD64-directed immunotherapy. An overview. Cancer Immunol. Immunother., *45:* 210–215, 1997.
47. Weiner, L. M., Clark, J. I., Davey, M., Li, W. S., de Palazzo, I. G., Ring, D. B., and Alpaugh, R. K. Phase I trial of 2B1, a bispecific monoclonal antibody targeting c-erbB-2 and FcγRIII. Cancer Res., *55:* 4586–4593, 1995.
48. Hartmann, F., Renner, C., Jung, W., Deisting, C., Juwana, M., Eichentopf, B., Kloft, M., and Pfreundschuh, M. Treatment of refractory Hodgkin's disease with an anti-CD16/CD30 bispecific antibody. Blood, *89:* 2042–2047, 1997.
49. van de Winkel, J. G. J., Bast, B., and de Gast, G. C. Immunotherapeutic potential of bispecific antibodies. Immunol. Today, *18:* 562–564, 1997.

[CANCER RESEARCH 47, 830–840, February 1, 1987]

Two New Monoclonal Antibodies, Lym-1 and Lym-2, Reactive with Human B-Lymphocytes and Derived Tumors, with Immunodiagnostic and Immunotherapeutic Potential[1]

Alan L. Epstein,[2] Robert J. Marder, Jane N. Winter, Efstathios Stathopoulos, Feng-Ming Chen, John W. Parker, and Clive R. Taylor

Department of Pathology [A. L. E., E. S., F-M. C., J. W. P., C. R. T.], University of Southern California School of Medicine, Los Angeles, California 90033; and the Departments of Pathology [R. J. M.] and Medicine [J. N. W.], Northwestern University and Lakeside Veterans Administration Medical Center, Chicago, Illinois 60611

ABSTRACT

Two new monoclonal antibodies (Lym-1 and Lym-2), reactive with the cell surface of B-lymphocytes and derived tumors, have been produced using tumor cell nuclei preparations as immunogens. Specificity screens using live cell radioimmunoassay techniques with 52 well-characterized human lymphoma and leukemia cell lines showed that both Lym-1 and Lym-2 bound to cell lines of B-cell lineage but were unreactive with those of T-cell, myeloid, or erythroid derivation. The B-cell specificity of these reagents was confirmed on 36 lymphoma and 15 leukemia biopsy specimens by using immunoperoxidase or immunofluorescence techniques. Additionally, flow cytometric analysis of 22 lymphoma biopsies showed that the majority of B-cell tumors were Lym-1 and/or Lym-2 positive and that within a given biopsy, a high percentage of the malignant cell population was stained. In both the immunoperoxidase and flow cytometric studies, reactive T-cells or T-cell lymphomas were consistently negative with the exception of Hodgkin's disease tissues which, in some instances, showed a higher than expected positivity with Lym-1 and Lym-2. Approximately 40% of B-cell chronic lymphocytic leukemias were found to be positive with Lym-1 while 80% were positive with Lym-2. Immunoperoxidase staining of frozen sections of human lymphoid tissues showed that both Lym-1 and Lym-2 stained germinal center and mantle zone B-lymphocytes as well as interfollicular histiocytes. Flow cytometric analysis of normal peripheral blood demonstrated specific staining of B-cells which comprised approximately 8% of circulating lymphocytes. Immunoperoxidase staining of nonlymphoid human organs and tissues revealed weak reactivity of Lym-1 with surface colonic epithelium only. Consistent with these findings, 35 solid tumor cell lines of diverse nature were found unreactive with both Lym-1 and Lym-2. Although standard techniques have thus far failed to identify the antigen recognized by Lym-2, the membrane antigen which binds Lym-1 has been shown by immunoprecipitation and competitive radioimmunoassay studies to be a polymorphic variant of the HLA-Dr antigen. Solid-phase radioimmunoassay techniques have shown that the antigens recognized by Lym-1 and Lym-2 are not significantly modulated after antibody exposure nor shed into the circulation of lymphoma patients. Finally, using iodine-125 labeled preparations of purified Lym-1 and Lym-2, we have determined that both reagents have a relatively large number of antibody binding sites per tumor cell and increased avidity for lymphoma cells when compared to normal and reactive lymph node B-cells. Because of the B-cell specificity of these reagents, their increased avidity for lymphoma cells, and their chemical stability after radiolabeling procedures, Lym-1 and Lym-2 appear to be promising reagents for the immunodiagnosis and therapy of the human malignant lymphomas.

INTRODUCTION

Monoclonal antibodies to human B-cell antigens have been developed by a number of laboratories in the last several years. The majority of these reagents identify B-cell lineage-specific differentiation antigens such as CB2 (1); BL1, BL2, and BL3 (2, 3); BL7 (4); L22, L23, and L24 (5); HLB-1 (6); H-76 (7); B1 (8, 9); B2 (10); B4 (11); B5 (12); 4G7 (13); BA1 (14); HB-4 (15); anti-Y 29/55 (16); B532 (17); OKB1, OKB2, OKB4, and OKB7 (18); 41H.16 (19); and FMC7 (20). Others appear to be directed against lymphoma-specific antigens such as LM-26 and LM-155 (21), against Burkitt's lymphoma cells, or Epstein-Barr virus-transformed cell lines such as BB-1 (22) and BLA (23, 24) or against blast-associated antigens such as B-LAST-1 (25) and BLAST-2 (26). In addition, our laboratory has reported on three monoclonal antibodies, designated LN-1, LN-2, and LN-3 which are directed against B-cell antigens, but unlike similar reagents, retain their reactivity in B5-fixed, paraffin-embedded tissues and are therefore useful reagents for the immunodiagnosis of human lymphomas (27–29).

Most of these B-cell reagents have been shown by immunoperoxidase techniques to stain normal B-cells of reactive lymphoid tissues as well as lymphomas of B-cell derivation. Comparison studies by Hofman *et al.* (30), LeBien *et al.* (31), Marder *et al.* (28), Knowles *et al.* (32), and Gobbi *et al.* (33) have clearly shown that these monoclonal antibodies identify different antigens that are expressed on topographically distinct subsets of B-cells in human lymphoid tissues. Although originally thought to be B-cell specific, many of these reagents have now been shown to have reactivities in nonlymphoid tissues attributable to the presence of similar, if not identical, epitopes in related or different molecules (34). Furthermore, a thorough analysis of these monoclonal antibodies to determine their clinical utility has not been performed to date. Evaluation of these reagents with respect to their binding reactivity with a wide spectrum of human tumor cell lines and biopsy specimens, avidity constants and antibody binding site number, presence or absence of antigenic modulation and antigen shedding, antibody stability after purification and chemical conjugation procedures, and biodistribution in tumor-bearing hosts are required to identify those monoclonal antibodies destined for clinical trial. Despite the large number of anti-B-cell monoclonal antibodies described thus far, a single monoclonal antibody with immunotherapeutic potential has not been identified.

In this report, we describe the development and characterization of two new B-cell specific monoclonal antibodies, designated Lym-1 and Lym-2, which after careful analysis, appear to have the necessary properties to be successful *in vivo* immunodiagnostic and immunotherapeutic reagents. Other related studies describing the biodistribution of Lym-1 and Lym-2 in tumor-bearing nude mice and volunteer lymphoma patients, the enhancement of NMR[3] spin echo imaging of tumors in animal hosts, and the results of clinical trials involving the immunotherapy and radioimmunotherapy of end-stage lym-

Received 4/22/86; revised 10/28/86; accepted 10/30/86

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by USPHS Grants R01-CA30621 and R01-CA40608 awarded by the National Cancer Institute, Department of Health and Human Services.

[2] To whom requests for reprints should be addressed, at Department of Pathology, University of Southern California School of Medicine, 2025 Zonal Avenue, Los Angeles, CA 90033.

[3] The abbreviations used are: NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; cpm, counts per minute; BSA, bovine serum albumin.

phoma patients are being completed and will be presented elsewhere.

MATERIALS AND METHODS

Cell Lines and Tissues

A complete list of the cell lines used in these experiments is shown in Tables 1–3. Tumor cell lines designated with the SW prefix were obtained from Dr. William B. McCombs, III, at the Scott and White Memorial Hospital and Scott, Sherwood, and Brinkley Foundation, Temple, TX. All of the cell lines were grown in RPMI 1640 medium containing 15% fetal calf serum, penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml). The cell lines were cultured in a well-humidified 5% CO_2 incubator and were routinely passaged twice weekly.

For the immunohistochemical and flow cytometric studies, human tissues were obtained from biopsies performed on patients for diagnostic procedures at Northwestern University Memorial Hospital, Chicago, IL, and Los Angeles County–University of Southern California Medical Center, CA. Tissues for the frozen section studies were immediately snap frozen in liquid nitrogen and stored at −70°C until sectioning. Tissues for paraffin embedding were fixed in 10% buffered formalin or B5 formalin by using standard procedures. Tissues for flow cytometry were obtained either from normal volunteers for the peripheral blood experiments or from lymphoma patients undergoing diagnostic procedures.

Generation and Characterization of Hybridoma Clones

The methods for antigen preparation, immunization, cell fusion and cloning, and serological characterization of monoclonal antibody isotypes have been previously described in detail (27).

Immunofluorescence Techniques

Live cell indirect immunofluorescence techniques used in these experiments have been described previously (27).

Immunoperoxidase Staining Methods

Frozen and paraffin sections were prepared for immunoperoxidase staining procedures as described previously (27). For these experiments, a 1/2 dilution of Lym-1 and Lym-2 supernatants were used.

Flow Cytometric Studies

Lym-1 and Lym-2 were studied for their binding reactivity to human peripheral blood and malignant lymph node mononuclear cells by flow cytometric techniques. Lym-1 and Lym-2 binding to peripheral blood lymphocytes was assessed using indirect immunofluorescence staining with biotinylated anti-mouse IgG followed by phycoerythrin-conjugated avidin staining. Identification of B- and T-lymphocytes was performed using fluorescein isothiocyanate-conjugated B1 and OKT11 (Ortho) monoclonal antibodies, respectively, after completion of the phycoerythrin indirect labeling procedures with Lym-1 and Lym-2. This allowed the simultaneous assessment of Lym-1 and Lym-2 staining with anti-B (B1) or anti-T (OKT11) reagents with known reactivity (8, 35). The results were obtained using dual color flow cytometric techniques with a 525-nm band pass filter for the green photomultiplier tube and a 590-nm long pass filter for the red photomultiplier tube. Monocytes were assessed using phycoerythrin-conjugated Leu M3 (Becton Dickinson) (36) to establish the number of monocytes in the lymphocyte gate as well as the location of the monocyte population on forward angle and log90° light scatter modes. Lym-1 and Lym-2 binding reactivity was determined on both the monocyte and lymphocyte gates independently.

Malignant lymph nodes obtained at the time of surgery from 22 adult patients were prepared into single cell suspensions by passing the gently minced tissue through a stainless steel mesh screen. After Ficoll-Hypaque gradient centrifugation separation, the cells were stained by live cell indirect immunofluorescence techniques as previously described (37). The cells were analyzed cytofluorometrically by using an EPICS C (Coulter Corp., Hialeah, FL) flow cytometer.

Purification of Lym-1 and Lym-2 for Iodination Procedures

For the radioimmunoassay studies described below, it was necessary to obtain microgram quantities of both Lym-1 and Lym-2. Since highly purified reagent was necessary for the iodination procedures, it was decided to use tissue culture supernatant instead of ascites fluid as the source of Lym-1 and Lym-2. For Lym-1, Protein A-Sepharose affinity chromatography was the method of choice since Protein A has been shown to bind well to mouse IgG_{2a} (38). Consequently, 1 liter of filtered spent supernatant was mixed continuously overnight at 4°C with 1 g of preswollen Protein A-Sepharose CL-4B (Pharmacia, Inc., Piscataway, NJ). The beads were then washed extensively with phosphate buffered saline (8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.1 g $CaCl_2 \cdot 2H_2O$, and 0.1 g $MgCl_2 \cdot 6H_2O$/liter of distilled water) by centrifugation and aspiration to remove unbound material. After next placing the beads into a small column, the Lym-1 antibody was eluted into 1-ml fractions with 0.05 M sodium acetate and 0.15 M NaCl, pH 4.3. Fractions containing eluted antibody were determined by optical density at 280 nm. After pooling the fractions containing antibody, the preparation was dialyzed overnight against a ×1000 volume of PBS[5] at 4°C. The antibody was then sterile filtered and stored in aliquots (1 mg/ml) at 4°C.

The IgG_1 Lym-2 monoclonal antibody was purified from 1 liter of filtered spent supernatant batchwise as described above but with 1 g of Affi-gel Protein A and MAPS buffers as described by Biorad, Inc. After elution, the Lym-2 monoclonal antibody was dialyzed overnight at 4°C against a ×1000 volume of PBS, sterile filtered, and stored in aliquots (1 mg/ml) at 4°C.

The purity of the Lym-1 and Lym-2 preparations was checked by 10% polyacrylamide gel electrophoresis and by Ouchterlony immunodiffusion using immunoglobulin heavy chain specific antisera (Miles, Elkhart, IN). Protein concentrations of the purified preparations were determined by optical density at 280 nm and by the Biorad Protein Assay Kit (Biorad).

Radioimmunoassay Methods

Live Cell Radioimmunoassay. Human malignant lymphoma, leukemia, and solid tumor cell lines were assessed for Lym-1 and Lym-2 binding using a live cell radioimmunoassay method. Briefly, suspension cultures and solid tumor cell lines which were dislodged from their flasks with EDTA-trypsin (Grand Island Biological Co., Grand Island, NY), were washed twice in cold buffer consisting of PBS, bovine serum albumin (1 mg/ml, radioimmunoassay grade; Sigma Chemical Co., St. Louis, MO), and 0.02% sodium azide. Cells (5×10^5) resuspended in 100 μl of wash buffer were pipeted into microtiter wells (Immulon Removawell Strips; Dynatech Laboratories, Inc., Alexandria, VA). The microtiter plates were pretreated the previous night with bovine serum albumin (10 mg/ml) in PBS with azide in order to prevent the antibody solutions from binding to the wells. Hybridoma supernatant (100 μl) was added to each well, and the plates were incubated for 30 min at room temperature with continuous mixing using a microshaker apparatus (Dynatech) set at low speed. After incubation, the cells were washed 4 times with cold wash buffer by spinning the plates at 1,000 rpm for 5 min, aspirating the supernatants with a 12-tip micromatic manifold (Popper and Sons, Inc., New Hyde Park, NY), and resuspending the cells in 200 μl of wash buffer using a Titertek Multichannel pipet (Flow Laboratories, Inc., McLean, VA) and the microshaker apparatus. After completion of the washes, 100,000 cpm of [^{125}I]goat anti-mouse serum (Amersham Corp., Arlington Heights, IL) in a volume of 100 μl, were added to each well for an additional 30-min incubation period with continuous shaking. Finally, the cells were washed 4 times as above and the wells were counted in a gamma counter at 1-min intervals. All data were expressed as the mean cpm of triplicate samples minus that of the NS-1 supernatant control wells. For each test, the standard deviation of the triplicate cpm was calculated and, if found to be greater than 10% of the mean cpm, the assay was repeated.

Detection of Shed Antigen. The presence of shed antigen was detected

using a sensitive sandwich solid-phase radioimmunoassay developed in our laboratory. Purified Lym-1 or Lym-2 at a concentration of 1 mg/ml in PBS was added to flat-well Immulon plates using a 50-μl volume per well. After a 1-h incubation at room temperature, the wells were washed once to remove unbound antibody. All washes were performed with 200 μl of BSA (1 mg/ml) in PBS containing 0.02% sodium azide. The wells were then blocked for 30 min with BSA (10 mg/ml) in PBS, washed twice, and incubated with 50 μl of either target cell membrane preparations, target cell supernatant concentrated ×25 in B15 Minicon Concentrators (Amicon Corp., Danvers, MA), or serum samples from lymphoma patients. The target cell lines used were Raji for Lym-1, ARH-77 for Lym-2, and CEM T-acute lymphoblastic leukemia as the negative control. After a 1-h incubation at room temperature, the wells were washed 3 times and 100,000 cpm/well of [^{125}I]sheep anti-mouse reagent were added to each well in a volume of 50 μl/well. After a final 1-h incubation period at room temperature, the wells were washed 5 times and counted in a gamma counter.

Antigenic Modulation. One million Raji cells (for Lym-1) or ARH-77 cells (for Lym-2) were incubated at time 0 with 100 μl of increasing concentrations of purified unlabeled Lym-1 or Lym-2 for 30 min at room temperature with continuous mixing. After removing unbound antibody by centrifugation, the cells were incubated for 24 and 48 h in complete RPMI 1640 medium at 37°C in a 5% CO_2 humidified incubator at which time 100 μl of 10^5 cpm of [^{125}I]Lym-1 or [^{125}I]Lym-2 were incubated with the cells for 30 min at room temperature with continuous mixing. After three washes, to remove unbound reagent, the cells were counted in a gamma counter. Iodine-125-labeled Lym-1 and Lym-2 monoclonal antibodies were prepared by a modified chloramine-T method as described below.

Quantitation of Antibody Avidity and Binding Site Number

In order to determine the avidity constant and binding site number of Lym-1 and Lym-2, a live cell radioimmunoassay was performed using directly labeled antibody preparations. The binding constants and number of antibody binding sites were determined by the methods and equations described by Frankel and Gerhard (39). Purified Lym-1 and Lym-2 were radioiodinated using a modified chloramine-T method (40). Briefly, 50–100 μg of antibody in 50–100 μl was added to 400 μCi of iodine-125 (4 μl) (New England Nuclear Research Products, Boston, MA) in a test tube. Chloramine-T was then added to the mixture and the reaction vessel was covered and incubated at room temperature for 90 s with constant shaking. The ratios of chloramine-T:Lym-1 and chloramine-T:Lym-2 were 1:4.0 and 1:4.6 (w:w), respectively. At the end of the incubation, the reaction was terminated by adding sodium metabisulfate at a 2:1 molar ratio to chloramine-T. The iodinated antibody was then separated from free iodine by standard methods using gel filtration. Fractions of labeled product were stored at 4°C until use. The protein concentration of the radiolabeled antibodies were determined by the BioRad Protein Assay using bovine serum albumin as the standard. Immunoreactivity defined as the percentage of labeled antibody still capable of binding to the target cell lines was assessed by live cell radioimmunoassay described above.

For the binding constant experiments, the concentration of antibody was varied by dilution with PBS. Each experimental variable was run in triplicate. Washed target cell suspensions containing 5×10^5 cells were added to each well of a 96-well Immulon microtiter plate which was previously blocked overnight with BSA (10 mg/ml) in PBS. The cells were incubated with 200 μl of PBS containing 0.5–500 ng of radiolabeled Lym-1 or Lym-2 for 1 h at room temperature with constant shaking. Cells were then washed ×3 with PBS containing bovine serum albumin (1 mg/ml) to remove unbound antibody and counted in a gamma counter. The amount of bound antibody was then determined by the radioactivity (cpm) in each well and the specific activity (cpm/ng) of the radiolabeled monoclonal antibody. For comparative purposes, parallel experiments using freshly prepared tonsil lymphocytes were also performed. The tonsil mononuclear cell preparation was prepared in the same manner as the malignant lymph nodes described above.

Live Cell Competition Studies. Competitive live cell radioimmunoassay procedures were performed with Lym-1 and anti-Dr and anti-Ds monoclonal antibodies (Mallinckrodt, Inc., St. Louis, MO) in order to determine the relationship of Lym-1 to HLA class II-related antigens expressed on human lymphoma cells. For these studies, 10^6 Raji cells per tube were washed twice with BSA (1 mg/ml) in PBS containing 0.02% sodium azide. The cells were then incubated at 4°C with 200 μl/tube of serial dilutions of anti-Dr and -Ds monoclonal antibodies for 30 min with constant mixing. After incubation, the cells were washed twice and 100,000 cpm/tube of [^{125}I]Lym-1 in a volume of 100 μl/tube were added for an additional 30-min incubation period. The cells were then washed twice and counted in a gamma counter.

Immunoprecipitation and Immunoblot Studies

Raji cells and ARH-77 myeloma cells were used in immunoprecipitation and immunoblot studies as previously described (27, 41) in order to identify the molecular weight of the antigens recognized by Lym-1 and Lym-2.

RESULTS

Generation of Monoclonal Antibodies Lym-1 and Lym-2. Hybridoma clone Lym-1 was produced by the fusion of mouse myeloma NS-1 cells and BALB/c splenocytes obtained from a mouse hyperimmunized with nuclei from Raji Burkitt's lymphoma cells. Isotypic analysis revealed that monoclonal antibody Lym-1 is of the IgG_{2a} heavy chain subclass. Hybridoma clone Lym-2 was produced as above from a mouse hyperimmunized with chronic lymphocytic leukemia cell nuclei. Isotypic analysis of supernatant revealed that Lym-2 is of the IgG_1 heavy chain subclass. Both monoclonal antibodies were initially identified by indirect immunofluorescence techniques with the use of paraformaldehyde-acetone-fixed cell preparations where they gave a speckled membrane (Lym-1) or membrane ring (Lym-2) pattern of staining. Both Lym-1 and Lym-2 have been subcloned on agar plates and have retained their ability to secrete antibody (2–10 μg/ml) in a stable manner for more than 2 yr. Both hybridomas have been adapted to grow exponentially in serum-free medium (Nutraclone-M; Techniclone International, Inc., Santa Ana, CA) with the same (Lym-1) or slightly slower (Lym-2) growth rates as cultures grown in 10% fetal calf serum facilitating their use in fermentation or hollow fiber culture equipment.

Binding Reactivity of Lym-1 and Lym-2 with Established Human Neoplastic Cell Lines by Live Cell Radioimmunoassay. The binding reactivities of monoclonal antibodies with established human malignant lymphoma, leukemia, and solid tumor cell lines are shown in Tables 1, 2, and 3, respectively. For these studies, a live cell radioimmunoassay method was used in order to directly assess the cell surface binding reactivity of these monoclonal antibodies with a wide spectrum of tumor cell lines. Both monoclonal antibodies appear unrestricted in their reactivity to human lymphomas and leukemias of B-cell origin.

Immunoperoxidase Staining Reactivity of Lym-1 and Lym-2 with Human Lymphoid and Non-Lymphoid Tissues. In order to assess the binding reactivity of Lym-1 and Lym-2 with normal human tissues, frozen sections of lymphoid and nonlymphoid organs from surgical biopsy material were stained with Lym-1 and Lym-2 supernatants by the avidin-biotin immunoperoxidase technique. As shown in Table 4 and in Fig. 1, both monoclonal antibodies reacted with the cell membrane of B-lymphocytes in human lymph node sections. Lym-1 showed a speckled membrane pattern and reacted principally with germinal center and mantle zone B-cells and interdigitating histiocytes scattered in the T-cell zones. Lym-2 gave a membrane ring pattern of staining and was positive on both germinal

Table 1 *Reactivity of Lym-1 and Lym-2 with human malignant lymphoma cell lines by live cell radioimmunoassay*

Cell line	Lym-1	Lym-2
Burkitt's lymphoma		
Raji	++++[a]	++
EB3	–	–
DG-75	++++	++++
NK-9	++	++++
AL-1	–	+
Daudi	+	+++
NU-AmB-1	+	++
SU-AmB-1	–	+
SU-AmB-2	–	–
RAMOS	–	–
Chevallier	++++	–
B46M	+	+
B35M	++++	++++
DND-39	+	–
U-698-M	+	++
HRIK	–	+
Large cell lymphoma		
SU-DHL-1	–	–
SU-DHL-2	–	–
SU-DHL-4	–	++++
SU-DHL-5	+	++
SU-DHL-6	+++	+++
SU-DHL-7	+	–
SU-DHL-8	+	–
SU-DHL-9	+	+
SU-DHL-10	–	++++
SU-DHL-16	–	–
NU-DHL-1	++++	–
U-937	–	–
Undifferentiated lymphoma		
NU-DUL-1	–	+

[a] –, <2,000 cpm; +, 2,000–6,000 cpm; ++, 6,000–10,000 cpm; +++, 10,000–15,000 cpm; ++++, >15,000 cpm.

Table 2 *Reactivity of Lym-1 and Lym-2 with human leukemia and lymphoblastoid cell lines by live cell radioimmunoassay*

Cell line	Lym-1	Lym-2
Acute lymphoblastic leukemia		
T-cell		
MOLT-4	–[a]	–
CEM	–	–
HSB-2	–	–
HPB-ALL	–	–
JM	–	–
Null cell		
REH	+	++
NALL-1	–	–
KM-3	–	–
L92-221	–	–
Pre-B-cell		
NALM-1 (from CML)	–	–
NALM-6	+	–
BALM-2	+++	++
BALM-5	–	–
B cell		
BALL-1	+++	+
Myeloid leukemia		
HL-60 (promyelocytic)	–	–
ML-2 (myeloid)		–
KG-1 (myeloid)	–	–
TPH-1-0 (monocytic)	–	–
K562 (erythroid CML)	–	–
HEL 92.1.7 (erythroid)	–	–
Myeloma		
U-266	–	–
ARH-77	+++	++++
HS Sultan	+++	+++
Lymphoblastoid		
BL-1	–	+
NU-LB-1	+++	++++
NU-LB-2	++	++
ERIC-LB-2	+++	+
ERIC-LB-3	+	++

[a] –, <2,000 cpm; +, 2,000–6,000 cpm; ++, 6,000–10,000 cpm; +++, 10,000–15,000 cpm; ++++, >15,000 cpm.

center and mantle zone B-lymphocytes. T-lymphocytes, sinus histiocytes, and endothelial cells of reactive lymph nodes and tonsils were not stained by both Lym-1 and Lym-2. In the thymus, Lym-1 was found to react with medullary dendritic cells, and in the spleen, both reagents stained B-cells of the white pulp. In multiple samples of normal bone marrow, both Lym-1 and Lym-2 were negative with myeloid, erythroid, and megakaryocyte precursor and mature cells.

As shown in Table 5, both Lym-1 and Lym-2 did not demonstrate significant binding reactivity with a large panel of normal human tissues obtained at biopsy. On some but not all specimens of human colon, Lym-1 did show weak staining reactivity with the epithelium of the luminal surface. Lym-1 was also positive with macrophages in the skin. Lym-2 was found completely unreactive with all tissues tested to date.

Staining Reactivity of Lym-1 and Lym-2 with Human Malignant Lymphoma and Leukemia Biopsy Specimens. As shown in Table 6, Lym-1 and Lym-2 were reacted with frozen sections from 36 lymphomas obtained at biopsy. Immunoperoxidase staining with Lym-1 and Lym-2 demonstrated significant and strong positivity with the majority of B-lymphomas. The T-cell lymphomas were unreactive with both antibodies even though they both were positive for the HLA-Dr antigen (data not shown). Lym-1 showed greater positivity with the large cell lymphomas and less reactivity with the small lymphocytic lymphomas while Lym-2 was found to react equally well with all types of B-lymphomas. An example of the immunoperoxidase staining reactivities of Lym-1 and Lym-2 on lymphoma tissue is presented in Fig. 2. As shown, this intermediate-grade lymphoma biopsy specimen had stronger reactivity with Lym-2 than Lym-1.

Lym-1 and Lym-2 were also reacted with 15 cases of chronic lymphocytic leukemia. As shown in Table 6, Lym-1 had a 40% reactivity with B-cell cases and no reactivity with all five T-cell chronic lymphocytic leukemias. Lym-2 showed an 80% reactivity with B-cell cases and likewise was unreactive with T-cell leukemias. These results indicate that both Lym-1 and Lym-2 are reactive with the majority of B-cell lymphomas and leukemias and are restricted in their binding to B-cell derived tumors.

Flow Cytometric Analysis of Lym-1 and Lym-2 Reactivity with Peripheral Blood Cells and Malignant Lymph Node Biopsy Specimens. In order to assess in a quantitative manner the type and percentage of Lym-1 and Lym-2 positive cells in normal peripheral blood mononuclear cells, flow cytometric studies were performed. In all cases studied, Lym-1 and Lym-2 were found unreactive with T-cells, Leu M3 positive monocytes, granulocytes, red blood cells, and platelets. B-lymphocytes, however, were found reactive with both Lym-1 and Lym-2 as demonstrated in Fig. 3 where dual labeling studies with the pan-B monoclonal antibody B1 showed simultaneous B1 and Lym-1 (Fig. 3*C*) and B1 and Lym-2 (Fig. 3*F*) reactivity. Quantitation of these results shows that for Lym-1, 90% of $B1^+$ cells are $Lym\text{-}1^+$ while 10% of $B1^+$ cells are $Lym\text{-}1^-$. For Lym-2, 86% of $B1^+$ cells are $Lym\text{-}2^+$ while 14% of $B1^+$ cells are $Lym\text{-}2^-$. Conversely, 10% of $B1^-$ cells are $Lym\text{-}1^+$ and 1% of $B1^-$ cells are $Lym\text{-}2^+$. These results indicate that roughly 8% of circulating B-lymphocytes found in normal peripheral blood samples are both Lym-1 and Lym-2 positive. The reactivities of monoclonal antibodies B1, Lym-1, and Lym-2, however, do show some differences indicating that small subpopulations of B-cells have varying reactivities with these B-cell reagents. As

Table 3 *Reactivity of Lym-1 and Lym-2 with 35 human solid tumor cell lines by live cell radioimmunoassay*

Cell line	Derivation	Lym-1	Lym-2
734B	Breast carcinoma	–[a]	–
578T	Breast carcinoma	–	–
C-399	Colon carcinoma	–	–
Hutu-80	Colon carcinoma	–	–
HT-29	Colon carcinoma	–	–
HeLa	Cervical carcinoma	–	–
SW 733	Papillary carcinoma of bladder	–	–
SW 780	Transitional cell carcinoma of bladder	–	–
SW 451	Squamous cell carcinoma of esophagus	–	–
SW 579	Squamous cell carcinoma of thyroid	–	–
SW 156	Hypernephroma	–	–
60	Small cell carcinoma of lung	–	–
464	Small cell carcinoma of lung	–	–
NCI-H69	Small cell carcinoma of lung	–	–
125	Adenocarcinoma of lung	–	–
A427	Adenocarcinoma of lung	–	–
A549	Adenocarcinoma of lung	–	–
SW 1503	Mesothelioma	–	–
BM 166	Neuroblastoma	–	–
IMR-5	Neuroblastoma	–	–
Y79	Retinoblastoma	–	–
A172	Astrocytoma	–	–
SW 608	Astrocytoma	–	–
U118 MG	Glioblastoma	–	–
NU-04	Glioblastoma	–	–
CaCl 74-36	Melanoma	–	–
Colo 38	Melanoma	–	–
SW 872	Liposarcoma	–	–
HS 919	Liposarcoma	–	–
SW 1045	Synovial sarcoma	–	–
SW 80	Rhabdomyosarcoma	–	–
SW 1353	Chondrosarcoma	–	–
4-998	Osteogenic sarcoma	–	–
4-906	Osteogenic sarcoma	–	–
SU-CCS-1	Clear cell sarcoma	–	–

[a] –, <2,000 cpm; +, 2,000–6,000 cpm; ++, 6,000–10,000 cpm; +++, 10,000–15,000 cpm; ++++, >15,000 cpm.

Table 4 *Reactivity of Lym-1 and Lym-2 with lymphoid and hematopoietic tissues in frozen sections or cytospins*

Organ	Lym-1	Lym-2
Lymph node		
Germinal center	+++[a]	++
Mantle zone	+	+++
T-cell zones	–	–
Interdigitating histiocytes	++	++
Sinus histiocytes	–	–
Endothelium	–	–
Thymus		
Cortex	–	–
Medulla	++ Dendritic cells	–
Spleen		
White pulp	++ B-cell zones	++ B-cell zones
Red pulp	–	–
Bone marrow		
Myeloid	–	–
Erythroid	–	–
Megakaryocytes	–	–

[a] Intensity of immunoperoxidase staining ranging from – to +++.

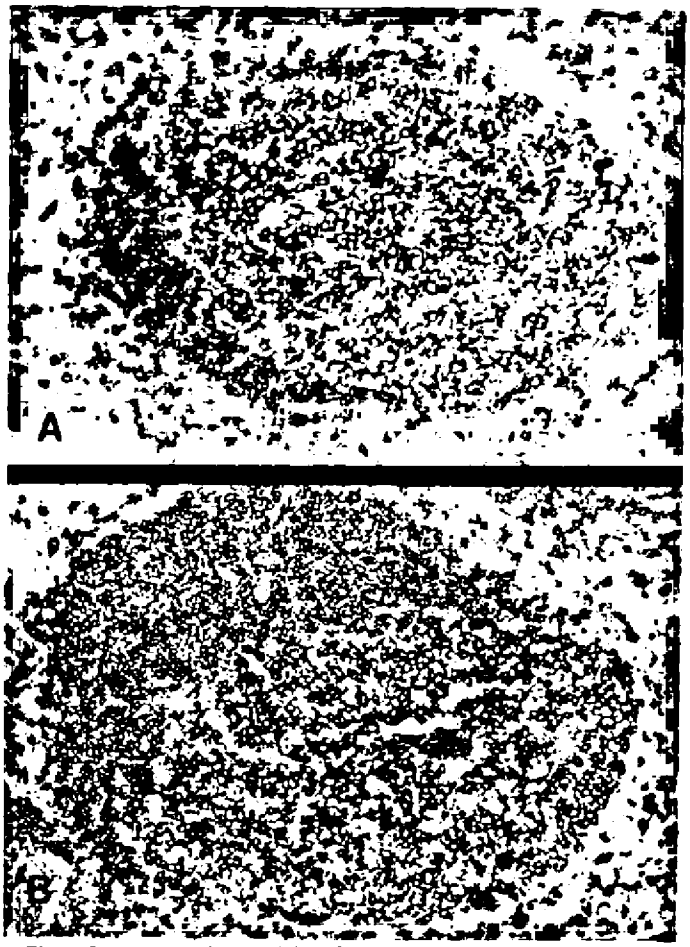


Fig. 1. Immunoperoxidase staining of Lym-1 and Lym-2 monoclonal antibodies with germinal center and mantle zone B lymphocytes in frozen sections of human tonsil. *A*, Lym-1; *B*, Lym-2 (× 325).

Table 5 *Reactivity of Lym-1 and Lym-2 with normal nonlymphoid tissues in frozen sections*

Tissue	Reactivity	
	Lym-1	Lym-2
Adrenal	–[a]	–
Brain	–	–
Breast	–	–
Cervix	–	–
Colon	+ surface epithelium	–
Duodenum	–	–
Heart	–	–
Kidney	–	–
Liver	–	–
Lung	–	–
Ovary	–	–
Pancreas	–	–
Salivary glands	–	–
Skin	+ macrophages only	–
Skeletal muscle	–	–
Smooth muscle	–	–
Stomach	–	–
Testis	–	–
Thyroid	–	–

[a] Intensity of immunoperoxidase staining ranging from – to +++.

shown in Table 7, flow cytometric analysis of 22 consecutive malignant lymphoma biopsy specimens revealed that Lym-1 and Lym-2 stained the majority of B-cell cases. OKT11 positive T-cell lymphomas (*cases 7* and *16*) were negative with both reagents. Lym-1 and Lym-2 showed similar but not identical staining reactivity and for individual cases, Lym-2 usually had a higher percentage of positive cells. For some of the Hodgkin's disease cases, such as *case 22*, there was an unusual overlapping of positivity with both Lym-1 and Lym-2 and OKT11, indicating that some T-cell marker positive cells were also expressing B-cell antigens. Further Hodgkin's disease cases need to be studied in order to determine the significance of these findings. In general, these data confirm the immunoperoxidase results reported above.

Radioimmunoassay Results of Antigen Shedding and Antigenic Modulation. The results of the antigen shedding and antigenic modulation studies are presented in Tables 8 and 9, respectively. These studies demonstrated that the antigen recognized by Lym-1 on human lymphoma cells is neither shed nor modulated after antibody binding. Sensitive solid-phase radioimmunoassay

Table 6 *Reactivity of Lym-1 and Lym-2 with human malignant lymphoma and leukemia biopsy specimens*

Diagnosis	Lym-1[a]	Lym-2[a]
Lymphomas[b] (frozen sections of lymph node biopsies[c])		
Well-differentiated lymphocytic	1/3	3/3
Poorly differentiated lymphocytic, nodular	0/2	2/2
Poorly differentiated lymphocytic, diffuse	1/3	3/3
Mixed lymphocytic and histiocytic	8/9	7/9
Histiocytic (B-cell)	12/17	12/17
T-cell	0/2	0/2
Leukemias (cytospins of peripheral blood[d])		
Chronic lymphocytic		
B-cell type	4/10	8/10
T-cell type	0/5	0/5

[a] Positive/total.
[b] Rappaport classification.
[c] Immunoperoxidase technique.
[d] Indirect immunofluorescence.

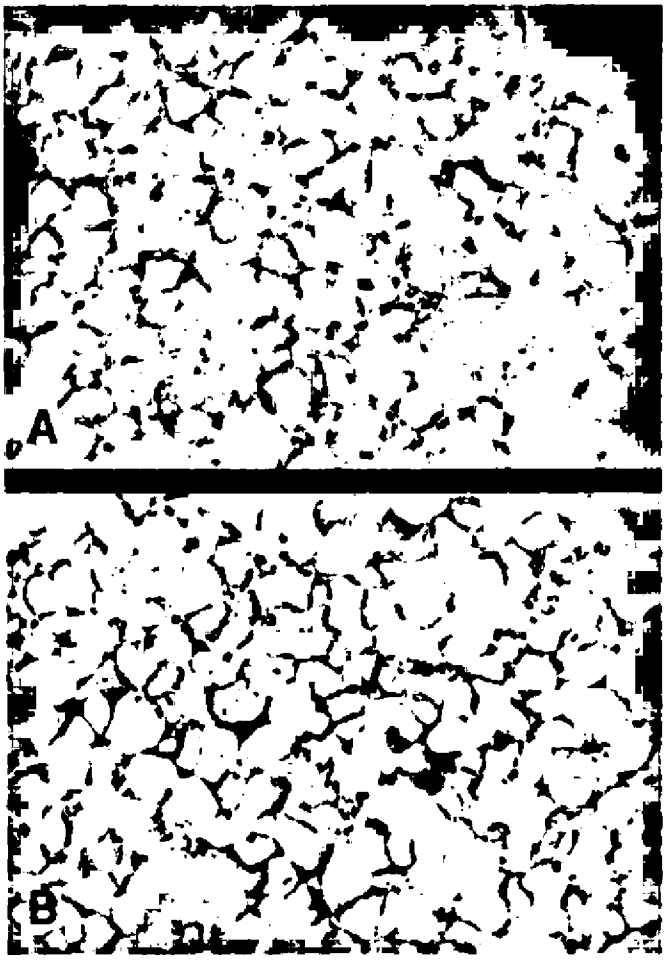


Fig. 2. Immunoperoxidase staining of Lym-1 and Lym-2 monoclonal antibodies with an intermediate grade lymphoma. *A*, Lym-1; *B*, Lym-2 (× 720).

methods also failed to detect circulating antigen in the serum of antigen positive lymphoma patients at the time of diagnosis. Although no antigen shedding was observed with Lym-2, partial modulation of the Lym-2 antigen was observed at high antibody concentrations (0.01 mg and higher).

Avidity Constant and Binding Site Number of Lym-1 and Lym-2 on Target Lymphoma Cell Lines and Reactive Tonsil B Lymphocytes. In Fig. 4, the binding profiles of radiolabeled Lym-1 and Lym-2 with Raji and ARH-77 tumor cells, respectively, are shown. With a large excess of antibody present, the equilibrium of the antigen-antibody reaction is expected to be driven in the direction of the antigen-antibody complex. Therefore, it is assumed that each determinant of antigen on the cell surface has bound with antibody at saturation. By this assumption, the number of binding sites per cell can be calculated by Equation A:

$$\frac{(\text{ng of Ab}) \times 10^9 \times N}{\text{Molecular weight of Ab} \times \text{number of cells}} \quad \text{(A)}$$

where N is Avogadro's Number and "ng of Ab" is the amount of antibody bound to the cell at saturation. The latter value was calculated from the cpm per well obtained by radioimmunoassay and the specific activity (cpm/ng) of the radiolabeled monoclonal antibody. Based upon these experiments, the number of binding sites per Raji Burkitt's lymphoma cell with Lym-1 was found to be 1.1×10^6, while the number of binding sites per ARH-77 cell with Lym-2 was 2.0×10^5.

As shown in Figs. 5 and 6, a Scatchard plot analysis of the radioimmunoassay data for Lym-1 and Lym-2 using Raji and ARH-77 cells, respectively, was used to obtain linear regression curves in order to calculate the slope. From the slope, the equilibrium or avidity constant K was calculated by the equation, $K = -(\text{slope}/n)$.

In Table 10 the avidity constants for Lym-1 and Lym-2 on target tumor cells lines and reactive tonsil lymphocytes are shown. A greater than 4-fold difference in K value for Lym-1 and approximately a 2-fold difference in K value for Lym-2 was obtained when tumor cell and tonsil lymphocytes were compared. These data indicate that both monoclonal antibodies have an increased avidity of binding for lymphoma cells as compared to normal B-lymphocytes.

Immunobiochemical Characterization of Lym-1 and Lym-2 Antigens. Immunoblot methods using denatured protein preparations failed to identify the molecular weight of the antigens recognized by Lym-1 and Lym-2. Immunoprecipitation techniques with metabolically labeled protein lysates did, however, identify the antigen recognized by Lym-1 as shown in Fig. 7. Using Raji cells as the source of radiolabeled proteins, 4 polypeptide bands with molecular weights of 31,000, 32,000, 34,000, and 35,000 were immunoprecipitated in a consistent manner by Lym-1 supernatant. Since this protein was so similar in molecular weight to the HLA-Dr antigen, side-by-side immunoprecipitation studies with Lym-1 and the anti-HLA-Dr monoclonal antibody SC2 (gift of Dr. Robert Fox, Scripps Clinic and Research Foundation) were performed. In Fig. 7, these immunoprecipitation results are shown with Raji cell lysates. The immunoprecipitation pattern obtained with Lym-1 was quite similar to that obtained with SC2 but differed in two respects. First, all of the bands identified in the SC2 immunoprecipitate are not seen in the Lym-1 immunoprecipitate indicating that Lym-1 only recognizes part of the HLA-Dr antigen or recognizes a polymorphic variant of the molecule. Second, a 3-week exposure for Lym-1 compared to a 10-day exposure for SC2 was required for the autoradiographs to be adequately visualized. These results may indicate that Lym-1 is seeing only a less abundant variant of the HLA-Dr molecule. To further test the relationship of Lym-1 to the HLA-Dr antigen, competition studies with Lym-1 and monoclonal anti-HLA-Dr and -Ds reagents were performed. As shown in Table 11, the anti-HLA-Ds monoclonal antibody was not able to block Lym-1 binding but the anti-HLA-Dr reagent successfully blocked Lym-1 binding to Raji cells even at low concentrations. These results confirm the immunoprecipitation results and suggest that Lym-1 is recognizing the HLA-Dr antigen or a closely related variant of this molecule.

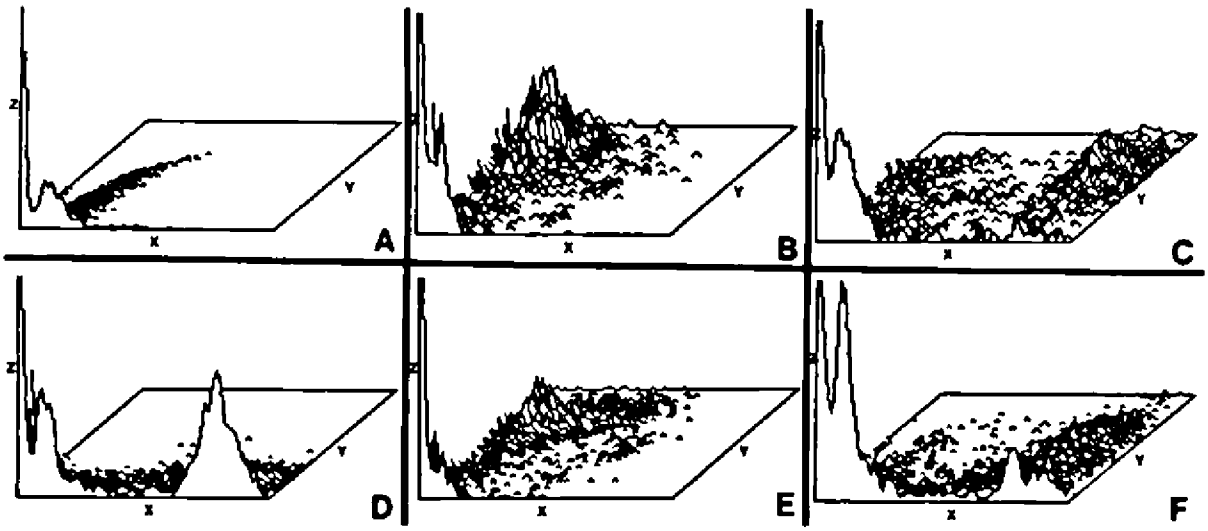


Fig. 3. Flow cytometric analysis of Lym-1 and Lym-2 staining reactivity with normal peripheral blood lymphocytes. *A*, negative control; *B*, Lym-1; *C*, Lym-1 and B1; *D*, B1; *E*, Lym-2, and *F*, Lym-2 and B1.

Table 7 *Flow cytometric analysis of malignant lymphoma biopsy specimens using Lym-1 and Lym-2 and lymphocyte markers*

Case	Sex	Age (yr)	Diagnosis (working formulation)	Biopsy site	OKT11	SIg	κ	λ	Lym-1	Lym-2
High grade lymphomas										
1	F	41	Small noncleaved, diffuse	L inguinal LN[a]	3	5	1	0	26	43
2	M	60	Small noncleaved, diffuse	R cervical LN	11	46	2	65	36	37
3	F	86	Small noncleaved, diffuse	Chest wall	26	67	36	0	2	0
4	M	47	Small noncleaved, diffuse	R inguinal LN	6	96	1	96	20	28
5	F	54	Immunoblastic	L submental LN	62	12	8	3	38	30
6	M	50	Immunoblastic	R axillary LN	55	82	18	18	80	70
7	F	89	Immunoblastic	L subclavian LN	96	25	21	1	12	17
Intermediate grade lymphomas										
8	F	73	Follicular, large cell	L axillary LN	18	87	87	7	57	84
9	F	31	Small cleaved, diffuse	Conjunctiva	8	95	7	85	51	75
10	M	87	Large cell, diffuse	R inguinal LN	14	23	9	1	86	61
Low grade lymphomas										
11	F	69	Follicular, small cleaved	L inguinal LN	16	38	2	34	76	80
12	M	53	Follicular, small cleaved	Small bowel	22	83	81	2	78	83
13	F	56	Follicular, small cleaved	L inguinal LN	26	100	79	2	85	90
14	F	39	Follicular, small cleaved	L inguinal LN	4	88	2	83	30	59
15	F	76	Follicular, mixed	R ovary	27	67	60	9	23	47
16	M	70	Follicular, large cell	L inguinal LN	61	10	7	5	8	31
17	M	55	Small lymphocytic (CLL)	Spleen	2	95	95	1	32	77
Hodgkin's disease										
18	M	38	HD/L-H	Cervical LN	39	72	63	64	69	86
19	F	15	NSHD	R cervical LN	81	20	17	8	7	24
20	F	18	NSHD	L inguinal LN	94	6	4	2	7	9
21	M	66	NSHD	L cervical LN	59	31	20	10	21	37
22	M	35	HD/mixed cellularity	R cervical LN	97	3	1	1	48	49

[a] LN, lymph node; HD, Hodgkin's disease; NSHD, nodular sclerosis Hodgkin's disease; HD/LH: Hodgkin's disease, lymphocytic-histiocytic type; L, left; R, right. CLL, chronic lymphocytic leukemia.

Immunoprecipitation studies with Lym-2 using ARH-77 radiolabeled lysates have to date yielded negative results. Additional experiments to identify the antigen recognized by Lym-2 are being conducted.

DISCUSSION

Two new monoclonal antibodies, Lym-1 and Lym-2, specific to normal and malignant human B-cells have been produced. The salient features of these antibodies are summarized in Table 12. Both monoclonal antibodies were developed from mice hyperimmunized with tumor cell nuclei as part of other experiments. Although these experiments were directed at producing reagents specific for nuclear constituents, we identified a handful of monoclonal antibodies with high avidity for the cell surface, which we assume recognized small remnants of cell

Table 8 *Detection of shed antigen by radioimmunoassay*

Antigen source	Radioimmunoassay	[^{125}I]Lym-1 (cpm)	[^{125}I]Lym-2 (cpm)
Target cells (1×10^6)	Live cell	51,582	40,105
CEM cells (1×10^6)	Live cell	214	456
Target membranes	Solid phase, direct	6,060	2,418
Target membranes	Solid phase, indirect	19,274	5,350
Target cell supernatant[a]	Solid phase, indirect	619	621
Lymphoma patient sera (no.)			
1	Solid phase, indirect	115	291
2	Solid phase, indirect	111	263
3	Solid phase, indirect	168	195
4	Solid phase, indirect	385	280
5	Solid phase, indirect	269	256

[a] Concentrated × 25.

Table 9 *Antigenic modulation studies with Lym-1 and Lym-2*

Concentration of Lym-1 and Lym-2 used to bind 10^6 target cells at 0 h	[^{125}I]Lym-1 (cpm)		[^{125}I]Lym-2 (cpm)	
	24 h	48 h	24 h	48 h
0.1 mg	18,444	32,938	3,662	4,850
0.05 mg	26,715	33,475	7,515	10,237
0.01 mg	31,502	40,169	11,008	17,105
0.005 mg	31,636	43,463	14,240	25,967
0.001 mg	37,665	46,928	19,776	27,116
PBS control	43,946	47,600	33,152	32,216

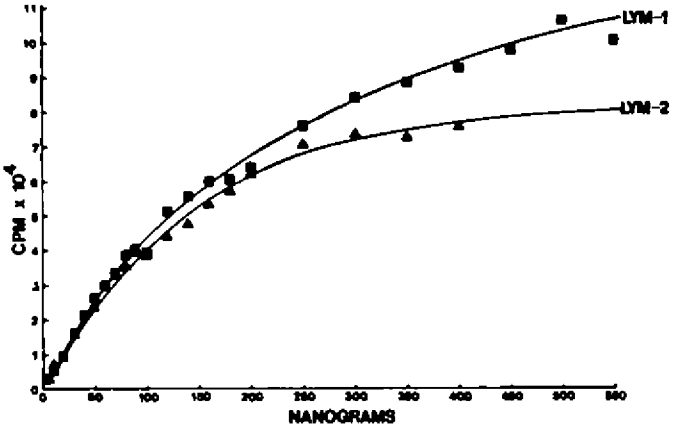


Fig. 4. Binding profiles of radiolabeled Lym-1 and Lym-2 reagents with Raji and ARH-77 cells, respectively.

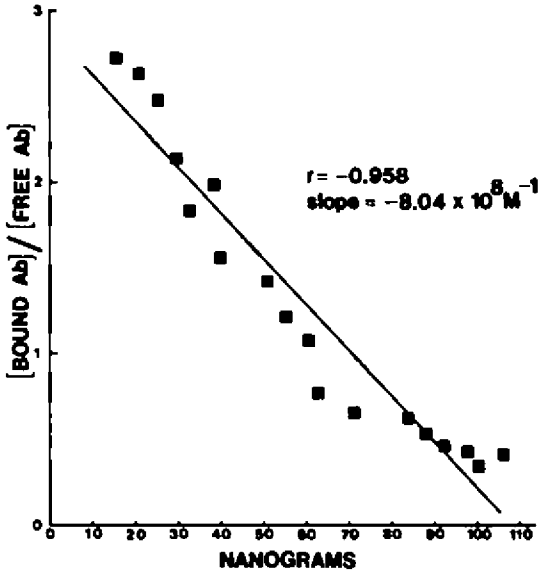


Fig. 5. Scatchard plot analysis of Lym-1 binding data with Raji cells.

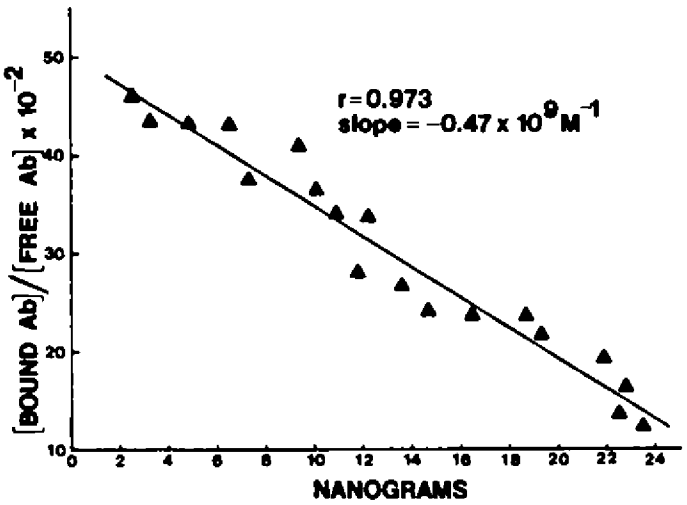


Fig. 6. Scatchard plot analysis of Lym-2 binding data with ARH-77 cells.

Table 10 *Avidity constants of Lym-1 and Lym-2 using target tumor cell lines and tonsil lymphocytes*

Monoclonal antibody	Tumor cell line	Tonsil
Lym-1	4.02×10^8 M^{-1}	0.88×10^8 M^{-1}
Lym-2	2.33×10^8 M^{-1}	1.23×10^8 M^{-1}

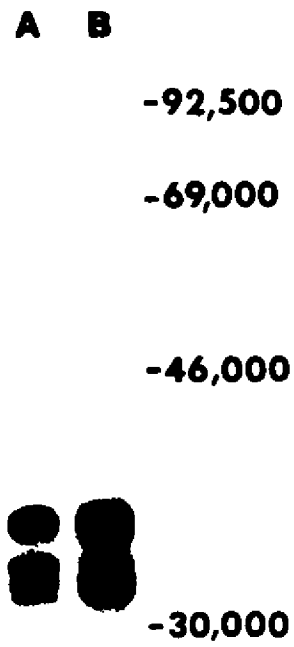


Fig. 7. Immunoprecipitation of [^{35}S]methionine- and [^{14}C]leucine-labeled Raji proteins by Lym-1 (*lane A*) and SC-2 anti-HLA-Dr (*lane B*) monoclonal antibodies.

membrane material in the nuclear preparations. Both Lym-1 and Lym-2 were such reagents and were initially identified using indirect immunofluorescence techniques using paraformaldehyde-acetone fixed cell preparations. In general, both monoclonal antibodies are reactive with circulating B lymphocytes and germinal center and mantle zone B-cells in reactive lymphoid tissues. Bone marrow and nonlymphoid tissues do not appear to be reactive with Lym-1 or Lym-2, with the exception of surface colonic epithelium which reacts weakly and variably with Lym-1 (Tables 4 and 5). Medullary dendritic cells of the thymus are also positive with Lym-1 but not with Lym-2. Both monoclonal antibodies react with surface membrane antigens which, in the case on Lym-1, has been shown by immunoprecipitation studies and competitive radioimmunoassay procedures to be related to the HLA-Dr antigen. Standard immunoprecipitation and immunoblot methods to identify the membrane antigen recognized by Lym-2 have thus far not been successful. Enzyme studies, however, have shown this antigen to be sensitive to proteases (data not shown) and further studies to identify its molecular weight are being conducted.

Lym-1 and Lym-2 are strongly reactive with the majority of B-cell derived malignancies, as demonstrated by their positive

Table 11 *Lym-1 competition studies with anti-HLA-Dr and -Ds monoclonal antibodies*

Competitive monoclonal antibody	Dilution [from stock solution (1 mg/ml)]	Bound [^{125}I]Lym-1 to 10^6 Raji cells (cpm)
Anti-HLA-Ds	None (PBS only)	3,464
	1:4	3,585
	1:8	3,101
	1:16	4,126
	1:32	2,901
	1:64	3,451
	1:128	3,397
	1:256	3,312
	1:512	3,496
	1:1,024	3,222
	1:2,048	3,946
	1:4,096	3,191
Anti-HLA-Dr	None (PBS only)	3,191
	1:4	453
	1:8	390
	1:16	531
	1:32	426
	1:64	443
	1:128	469
	1:256	1,109
	1:512	1,812
	1:1,024	2,304
	1:2,048	2,970
	1:4,096	4,061

binding to Burkitt's lymphoma, large cell lymphoma, B-cell acute lymphoblastic leukemia, and Epstein-Barr virus-transformed lymphoblastoid cell lines (Tables 1 and 2). T-cell acute lymphoblastic and myeloid leukemia cell lines as well as a wide spectrum of solid tumor cell lines (Table 3) were consistently negative by live cell radioimmunoassay procedures. Further studies using immunoperoxidase staining of frozen sections (Table 6) or immunofluorescence staining of dissociated lymphoma and leukemia biopsies using quantitative flow cytometric techniques confirmed the B-cell specificity of Lym-1 and Lym-2 (Table 7). Although Lym-1 was shown to be related to the HLA-Dr antigen, it did not bind to HLA-Dr-positive T-cell lymphomas or to activated endothelial cells and showed different, albeit similar, binding reactivities with lymphoma and leukemia cell lines when compared to standard HLA-Dr monoclonal antibody reagents. Because of these data, it is probable that Lym-1 recognizes a polymorphic variant of the HLA-Dr molecule which confers upon Lym-1 greater specificity to normal and malignant human B-cells. The binding reactivities of Lym-1 and Lym-2 shown in Tables 1 and 2 are significantly different for each other and for other published B-cell specific monoclonal antibodies, providing preliminary evidence that they recognize unique antigens expressed on human B-cells and derived malignancies. Direct comparison studies would be required to verify this point.

Radioimmunoassay methods using iodine-125-labeled Lym-1 and Lym-2 have demonstrated the presence of relatively high numbers of binding sites on target tumor cell lines. These data suggest that Lym-1 or Lym-2 conjugated to cytotoxic drugs may be effective as an alternative form of therapy since the number of binding sites per tumor cell is a critical factor in achieving significant drug concentrations at the tumor site (42). In addition, both monoclonal antibodies were shown to have high avidity binding constants in excess of 10^8 M^{-1}. Comparative studies with reactive B-cells from tonsil biopsies showed avidity binding constants with 4-fold and 2-fold lower values for Lym-1 and Lym-2, respectively, than those obtained with lymphoma target cell lines (Table 10). These data, which were obtained from measurements made independently of the binding site number, indicate that both Lym-1 and Lym-2 bind preferentially to lymphoma cells rather than to normal or reactive lymph node B-cells. The number of antibody binding sites per cell as anticipated was also higher on lymphoma cells (data not shown) which are generally larger and more homogeneous in size than reactive tonsil B-cells which range from small to large depending on their state of differentiation.

Sensitive solid-phase radioimmunoassay techniques revealed that both Lym-1 and Lym-2 recognize membrane antigens which are not shed from malignant cells grown in culture nor present free in the circulation of lymphoma patients (Table 8). Furthermore, the antigens recognized by Lym-1 and Lym-2 were not found to modulate significantly on the surface of target tumor cell lines after exposure to purified antibody preparations (Table 9).

As summarized in Table 13, Lym-1 and Lym-2 appear to have several advantages as immunodiagnostic and immunotherapeutic reagents. The data presented here suggest that these monoclonal antibodies would be useful imaging agents after conjugation with radioisotopes. As set forth by DeNardo *et al.* (43), cancer-seeking monoclonal antibodies carrying radionuclides can, in theory, be very powerful reagents for the radioimaging and radiotherapy of cancer. Optimization of this type of therapy requires dynamic modeling of physiological parameters which govern radionuclide distribution in the patient. Certain critical properties of the radiolabeled antibody must also be present in order for the target-to-nontarget uptake ratio to equate to favorable imaging and therapy. For Lym-1, the parameters for radioconjugation with radioactive iodine have been identified and experiments with this monoclonal

Table 12 *Characterization of monoclonal antibodies Lym-1 and Lym-2*

	Lym-1[a]	Lym-2[b]
Immunogen	Raji nuclei	CLL biopsy nuclei
Isotype	IgG_{2a}	IgG_1
Antigen	Protein[c]	Unknown
Antigen site	Cell surface	Cell surface
Number of antibody binding sites	1.1×10^6 sites/Raji cell	2×10^5 sites/ARH-77 cell
Antibody avidity constant	4.02×10^8 M^{-1}	2.33×10^8 M^{-1}
Lymphoid reactivity		
Lymph node and tonsil	B-cell zones and histiocytes	B-cell zones and histiocytes
Bone marrow	None	None
Blood	B-cells[d]	B-cells[d]
Thymus	Medullary dendritic cells	None
Spleen	B-cells	B-cells
Nonlymphoid reactivity	Surface colonic epithelium	None
Tumor specificity	B-cell lymphomas and leukemias	B-cell lymphomas and leukemias

[a] Studies performed with Raji Burkitt's lymphoma cells.
[b] Studies performed with ARH-77 myeloma cells.
[c] M_r 31,000, 32,000, 34,000, and 35,000.
[d] Represents 8% of total peripheral blood mononuclear cells.

Table 13 *Advantages of Lym-1 and Lym-2 as in vivo immunodiagnostic and immunotherapeutic reagents*

Antigen
Small antigen reservoir.
Not shed nor modulated.
High number of antigenic sites per tumor cell.
Expressed on majority of human lymphomas.
Antibody
Highly stable after chemical or isotope conjugation procedures.
High avidity binding constants.
2–4-fold increase in avidity binding constants with tumor cells compared to normal B-lymphocytes.
Favorable isotype (IgG_{2a} for Lym-1) for interaction with patient immune response.

antibody indicate high retention of label and immunoreactivity after conjugation procedures (44, 45). Based upon these studies, Lym-1 has been used successfully to image lymphoma lesions in the nude mouse and in volunteer patients after iodine-123 conjugation (46). Successful radioimmunotherapy has also been achieved in lymphoma-bearing nude mice treated with single doses of [^{131}I]Lym-1 preparations (47). Because of these results, radioimmunotherapeutic trials using [^{131}I]Lym-1 have been initiated at the University of California at Davis under the guidance of Drs. Sally and Gerald DeNardo. Early results from these trials suggest that this reagent may be a powerful radioimmunotherapeutic and imaging agent. Clinical trials with unlabeled Lym-1 and Lym-2 have also been initiated at the University of Southern California to test the effectiveness and toxicity, if any, of these reagents. Finally, Lym-1 has been used to enhance NMR spin echo imaging of lymphoma-bearing nude mice after conjugation with gadolinium.[4] All of these studies emphasize the possible utility of Lym-1 and Lym-2 for the radioimmunodetection and therapy of human lymphomas. Because of their B-cell specificity, high binding avidities for human lymphomas, and stability after chemical conjugation procedures, these reagents can play an important role in delineating the necessary parameters for the successful immunodiagnosis and immunotherapy of the human malignant lymphomas after linkage with radionuclides, NMR-enhancing agents, cytotoxic drugs and toxins, or immunologically reactive peptides.

ACKNOWLEDGMENTS

The authors wish to thank Drs. Sally and Gerald DeNardo for their intellectual input and support during the course of this work. The authors also want to thank Helen Salwen, Eileen Connelly, and Suzan Holland for their excellent technical assistance.

REFERENCES

1. Jephthah, J., Terasaki, P. I., Hofman, F., Wolde-Marian, W., Peter, J. B., and Billing, R. A cytotoxic monoclonal antibody detecting a novel B-cell membrane antigen expressed predominantly on cells bearing surface membrane immunoglobulin. Blood, *63:* 319–325, 1984.
2. Knowles, D. M., II, Tolidjian, B., Marboe, C. G., Halper, J. P., Azzo, W., and Wang, C. Y. A new human B lymphocyte surface antigen (BL2) detectable by a hybridoma monoclonal antibody: distribution on benign and malignant lymphoid cells. Blood, *62:* 191–199, 1983.
3. Wang, C. Y., Azzo, W., Al-Katib, A., Chiorazzi, N., and Knowles, D. M., II Preparation and characterization of monoclonal antibodies recognizing three distinct differentiation antigens (BL1, BL2, BL3). J. Immunol., *133:* 684–691, 1984.
4. Al-Katib, A., Wang, C. Y., and Koziner, B. Distribution of a new B-cell-associated surface antigen (BL7) detected by a monoclonal antibody in human leukemic disorders. Cancer Res., *45:* 3058–3063, 1985.
5. Takami, T., Ishii, Y., Yuasa, H., and Kikuchi, K. Three distinct antigen systems on human B-cell subpopulations as defined by monoclonal antibodies. J. Immunol., *134:* 828–834, 1985.
6. Kasai, K., Koshiba, H., Ishii, Y., and Kikuchi, K. A monoclonal antibody defining human B-cell differentiation antigen (HLB-1 antigen). Microbiol. Immunol., *27:* 51–64, 1983.
7. Gasser, D. L., Winters, B. A., Haas, J. B., McKearn, T. J., and Kennett, R. H. Monoclonal antibody directed to a B-cell antigen present in rats, mice and humans. Proc. Natl. Acad. Sci. USA, *76:* 4636–4640, 1979.
8. Nadler, L. M., Ritz, J., Hardy, R., Pesando, J. M., and Schlossman, S. F. A unique cell surface antigen identifying lymphoid malignancies of B cell origin. J. Clin. Invest., *67:* 134–140, 1981.
9. Stashenko, P., Nadler, L. M., Hardy, R., and Schlossman, S. F. Characterization of a human B lymphocyte-specific antigen. J. Immunol., *125:* 1678–1685, 1980.
10. Nadler, L. M., Stashenko, P., Hardy, R., van Agthoven, A., Terhorst, C., and Schlossman, S. F. Characterization of a human B-cell-specific antigen (B2) distinct from B1. J. Immunol., *126:* 1941–1947, 1981.
11. Nadler, L. M., Anderson, K. C., Marti, G., Bates, M., Park, E., Daley, J. F., and Schlossman, S. F. B4, a human B lymphocyte-associated antigen expressed on normal, mitogen-activated and malignant B lymphocytes. J. Immunol., *131:* 244–250, 1983.
12. Freedman, A. S., Boyd, A. W., Anderson, K. C., Fisher, D. C., Schlossman, S. F., and Nadler, L. M. B5, a new B-cell-restricted activation antigen. J. Immunol., *134:* 2228–2235, 1985.
13. Meeker, T. C., Miller, R. A., Link, M. P., Bindl, J., Warnke, R., and Levy, R. A unique human B lymphocyte antigen defined by a monoclonal antibody. Hybridoma, *3:* 305–320, 1984.
14. Abramson, C. S., Kersey, J. H., and LeBien, T. W. A monoclonal antibody (BA-1) reactive with cells of human B lymphocyte lineage. J. Immunol., *126:* 83–88, 1981.
15. Tedder, T. F., Clement, L. T., and Cooper, M. D. Development and distribution of a human B-cell subpopulation identified by the HB-4 monoclonal antibody. J. Immunol., *134:* 1539–1544, 1985.
16. Forster, H. K., Gudak, F. G., Girard, M.-F., Albrecht, R., Schmidt, J., Ladwig, C., and Obrecht, J.-P. Monoclonal antibody against a membrane antigen characterizing leukemic human B lymphocytes. Cancer Res., *42:* 1927–1934, 1982.
17. Frisman, D., Slovin, S., Royston, I., and Baird, S. Characterization of a monoclonal antibody that reacts with an activation antigen on human B cells: reactions on mitogen-stimulated blood lymphocytes and cells of normal lymph nodes. Blood, *62:* 1224–1229, 1983.
18. Mittler, R. S., Talle, M. A., Carpenter, K., Rao, P. E., and Goldstein, G. Generation and characterization of monoclonal antibodies reactive with human B lymphocytes. J. Immunol., *131:* 1754–1761, 1983.
19. Zipf, T. F., Lauzon, G. J., and Longenecker, B. M. A monoclonal antibody detecting a 39,000 M.W. molecule that is present on B lymphocytes and chronic lymphocytic leukemia cells but is rare on acute lymphocytic leukemia blasts. J. Immunol., *131:* 3064–3072, 1983.
20. Brooks, D. A., Beckman, I. G. R., Bradley, J., McNamara, P. J., Thomas, M. E., and Zola, H. Human lymphocyte markers defined by antibodies derived from somatic cell hybrids. IV. A monoclonal antibody reacting specifically with a subpopulation of human B lymphocytes. J. Immunol., *126:* 1373–1377, 1981.
21. Howard, D. R., Eaves, A. C., and Takei, F. Two monoclonal antibodies defining unique antigenic determinants on human B-lymphoma cells. Cancer Res., *45:* 6322–6327, 1985.
22. Yokochi, T., Holly, R. D., and Clark, E. A. B lymphoblast antigen (BB-1) expressed on Epstein-Barr virus-activated B cell blasts, B lymphoblastoid cell lines, and Burkitt's lymphomas. J. Immunol., *128:* 823–827, 1982.
23. Weils, J., Fellous, M., and Tursz, T. Monoclonal antibody against a Burkitt's lymphoma-associated antigen. Proc. Natl. Acad. Sci. USA, *78:* 6485–6488, 1981.
24. Weils, J., Lenoir, G. M., Fellous, M., Lipinski, M., Salomon, J. C., Tetaud, C., and Tursz, T. A monoclonal antibody with anti-Burkitt's lymphoma specificity. I. Analysis of human hematopoietic and lymphoid cell lines. Int. J. Cancer, *29:* 653–658, 1982.
25. Thorley-Lawson, D. A., Schooley, R. T., Bhan, A. K., and Nadler, L. M. Epstein-Barr virus superinduces a new human B-cell differentiation antigen (B-LAST-1) expressed on transformed lymphoblasts. Cell, *30:* 415–425, 1982.
26. Thorley-Lawson, D. A., Nadler, L. M., Bhan, A. K., and Schooley, R. T. BLAST-2 (EBVCS), an early cell surface marker of human B-cell activation, is superinduced by Epstein-Barr virus. J. Immunol., *134:* 3007–3012, 1985.
27. Epstein, A. L., Marder, R. J., Winter, J. N., and Fox, R. I. Two new monoclonal antibodies (LN-1, LN-2) reactive in B5 formalin-fixed, paraffin-embedded tissues with follicular center and mantle zone human B lymphocytes and derived tumors. J. Immunol., *133:* 1028–1036, 1984.
28. Marder, R. J., Variakojis, D., Silver, J., and Epstein, A. L. Immunohistochemical analysis of human lymphomas with monoclonal antibodies to B-cell and Ia antigens reactive in paraffin sections. Lab. Invest., *52:* 497–504, 1985.
29. Okon, E., Felder, B., Epstein, A. L., Lukes, R. J., and Taylor, C. R. Monoclonal antibodies reactive with B lymphocytes and histiocytes in paraffin sections. Cancer (Phila.), *56:* 95–104, 1985.
30. Hofman, F. M., Yanagihara, E., Byrne, B., Billing, R., Baird, S., Frisman, D., and Taylor, C. R. Analysis of B-cell antigens in normal reactive lymphoid tissues using four B-cell monoclonal antibodies. Blood, *62:* 775–783, 1983.
31. LeBien, T., Kersey, J., Nakazawa, S., Minato, K., and Minowada, J. Analysis of human leukemia/lymphoma cell lines with monoclonal antibodies BA-1, BA-2 and BA-3. Leuk. Res., *6:* 299–305, 1982.

[4] M. McNamara, University of California at San Francisco, NMR Institute, personal communication.

32. Knowles, D. M., II, Tolidjian, B., Marboe, C. C., Mittler, R. S., Talle, M. A., and Goldstein, G. Distribution of antigens by OKB monoclonal antibodies on benign and malignant lymphoid cells and on nonlymphoid tissues. Blood, *63:* 886–896, 1984.
33. Gobbi, M., Caligaris-Cappio, F., and Janossy, G. Normal equivalent cells of B-cell malignancies: analysis with monoclonal antibodies. Br. J. Hematol., *54:* 393–403, 1983.
34. Hsu, S-M., Zhang, H-Z., and Jaffe, E. S. Monoclonal antibodies directed against human lymphoid, monocytic, and granulocytic cells: reactivities with other tissues. Hybridoma, *2:* 403–412,1983.
35. Verbi, W., Greaves, M. F., Schneider, C., Koubeck, K., Janossy, G., Stein, H., Kung, P., and Goldstein, G. Monoclonal antibodies OKT11 and OKT11A have pan-T reactivity and block sheep erythrocyte "receptors." Eur. J. Immunol., *12:* 81–86, 1982.
36. Dimitriu-Bona, A., Burmester, G. R., Waters, S. J., and Winchester, R. J. Human mononuclear phagocyte differentiation antigens. I. Patterns of antigenic expression on the surface of human monocytes and macrophages defined by monoclonal antibodies. J. Immunol., *130:* 145–152, 1983.
37. Winter, J. N., Variakojis, D., and Epstein, A. L. Phenotypic analysis of established diffuse histiocytic lymphoma cell lines utilizing monoclonal antibodies and cytochemical techniques. Blood, *63:* 140–146, 1984.
38. Ey, P. L., Prowse, S. J., and Jenkin, C. R. Isolation of pure IgG, IgG_{2a}, and IgG_{2b} immunoglobulins from mouse serum using protein-A-sepharose. Immunochemistry, *15:* 429–436, 1978.
39. Frankel, M. E., and Gerhard, W. The rapid determination of binding constants for antiviral antibodies by a radioimmunoassay. An analysis of the interaction between hybridoma proteins and influenza virus. Mol. Immunol., *16:* 101–106, 1979.
40. McFarlane, A. S. Labelling of plasma proteins with radioactive iodine. Biochem. J., *62:* 135–143, 1956.
41. Epstein, A. L. Immunobiochemical characterization with monoclonal antibodies of Epstein-Barr virus-associated early antigens in chemically induced cells. J. Virol., *50:* 372–379, 1984.
42. Varki, N. M., Reisfeld, R. A., and Walker, L. E. Effect of monoclonal antibody-drug conjugates on the *in vivo* growth of human tumors established in nude mice. *In:* Monoclonal Antibodies and Cancer Therapy, pp. 207–214. New York: Alan R. Liss, Inc., 1985.
43. DeNardo, G. L., Raventos, A., Hines, H. H., Scheibe, P. O., Macey, D. J., Hays, M. T., and DeNardo, S. J. Requirements for a treatment planning system for radioimmunotherapy. Int. J. Radiat. Oncol. Biol. Phys., *11:* 335–348, 1985.
44. Mills, S. L., DeNardo, S. J., DeNardo, G. L., Epstein, A. L., Peng, J-S., and Colcher, D. I-123 radiolabelling of monoclonal antibodies for *in vivo* procedures. Hybridoma, *5:* 265–275, 1986.
45. DeNardo, S. J., Peng, J. B., DeNardo, G. L., Mills, S. L., and Epstein, A. L. Immunochemical aspects of monoclonal antibodies important for radiopharmaceutical development. Int. J. Nucl. Med. Biol., in press, 1986.
46. Epstein, A. L., Zimmer, A. M., Spies, S. M., Mills, S., DeNardo, G., and DeNardo, S. Radioimmunodetection of human B-cell lymphomas with a radiolabelled tumor-specific monoclonal antibody (Lym-1). *In:* F. Cavalli, G. Bonadonna, and M. Rozenceweig (eds.), Malignant Lymphomas and Hodgkin's Disease: Experimental and Therapeutic Advances, pp. 569–577. Boston: Martinus Nijoff Publ. Co., 1985.
47. Adams, D. A., DeNardo, G. L., DeNardo, S. J., Matson, G. B., Epstein, A. L., and Bradbury, E. M. Radioimmunotherapy of human lymphoma in athymic, nude mice as monitored by P-31 nuclear magnetic resonance. Biochem. Biophys. Res. Commun., *131:* 1020–1027, 1985.

These documents were entered into the record on February 4, 2008.

blood

2001 98: 1095-1099
doi:10.1182/blood.V98.4.1095

Therapeutic efficacy of intravenous immunoglobulin preparations depends on the immunoglobulin G dimers: studies in experimental immune thrombocytopenia

Jessica L. Teeling, Theo Jansen-Hendriks, Taco W. Kuijpers, Masja de Haas, Jan G. J. van de Winkel, C. Erik Hack and Wim K. Bleeker

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.

Therapeutic efficacy of intravenous immunoglobulin preparations depends on the immunoglobulin G dimers: studies in experimental immune thrombocytopenia

Jessica L. Teeling, Theo Jansen-Hendriks, Taco W. Kuijpers, Masja de Haas, Jan G. J. van de Winkel, C. Erik Hack, and Wim K. Bleeker

The clinical benefit of intravenous immunoglobulin (IVIG) preparations in the treatment of immune thrombocytopenic purpura (ITP) is supposed to be mediated by blockade of Fcγ receptor–bearing phagocytes. In 2 experimental models for ITP, it is shown that the therapeutic efficacy of IVIG preparations is related to the IgG dimer content present in these preparations. A rat monoclonal antibody (mAb; MWReg30) directed to the murine platelet-specific integrin $\alpha_{IIb}\beta_3$ (gpIIb/IIIa) was administered intraperitoneally either as bolus injection or continuous infusion. With bolus injection, the circulating platelet count dropped to almost zero within 3 hours. Pretreatment with cobra venom factor did not affect platelet depletion, whereas pretreatment with anti-FcγRII/III mAb 2.4G2 or IVIG greatly reduced platelet clearance. With continuous infusion, platelet numbers reached a steady state after 4 days, at approximately 25% of control. This reduction in platelets was, however, not observed in mice deficient for the FcRγ-chain, lacking FcγRI, FcγRIII, and FcγRIII$^{-/-}$ mice. Infusion of a single dose of IVIG with a high IgG dimer content on the 4th day—ie, mimicking therapeutic administration—resulted in a platelet increase for several days. IVIG predominantly consisting of monomeric IgG had no effect on platelet numbers. In conclusion, continuous infusion of MWReg30 induces thrombocytopenia in mice by enhancing Fcγ receptor–mediated clearance of platelets. In this model, it is shown that IgG dimers present in IVIG preparations are responsible for the increase in platelet counts. (Blood. 2001;98:1095-1099)

Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune disorder in which antibody-sensitized platelets are prematurely destroyed by phagocytic cells in the reticuloendothelial system (RES).[1] Therapeutic interventions in ITP are often directed toward reducing antibody-mediated clearance by either decreasing the production of pathogenic platelet autoantibodies or impairing RES function. Imbach et al[2] was the first to report that intravenous immunoglobulin (IVIG) preparations were effective in children with acute and chronic ITP. Administration of IVIG leads to rapid, yet often transient, reversal of thrombocytopenia in most patients with ITP.[3]

In the treatment of ITP, there may be multiple mechanisms of action of IVIG. It has been proposed that IVIG exerts its function though blockade of Fcγ receptors or neutralization of autoantibodies by idiotype–anti-idiotype interaction. However, clinical observations sometimes show prolonged effects of IVIG that cannot be explained by the short-term neutralization of pathologic antibodies or Fcγ receptor blockade. Therefore, other mechanisms, such as modulation of cytokine synthesis or interference with T- and B-cell function, have been suggested to explain long-term effects in the treatment of ITP by IVIG.[4]

Fcγ receptors play an important role in antibody-dependent clearance, and many studies have provided evidence that reversal of thrombocytopenia can be induced by (non)specific blockade of Fcγ receptors on phagocytes in the RES.[5] A predominant role for low-affinity Fcγ receptors in antibody-mediated thrombocytopenia has been shown in animal studies[6,7] and mice deficient in Fcγ receptors[8] or transgenic in mice overexpressing human FcγRIIA.[9] Treatment of a patient with chronic ITP with a monoclonal antibody against FcγRIII was reported to result in impressive, albeit transient, increases in platelet counts, supposing that the platelets in human ITP are also destroyed by binding to low-affinity Fcγ receptors.[10]

Three different types of murine FcγR exist: a high-affinity receptor, FcγRI (CD64), capable of affinity-binding monomeric IgG, and 2 low-affinity receptors, FcγRII (CD32) and FcγRIII (CD16), preferentially binding polymeric IgG or immune complexes. FcγRII is widely distributed on lymphoid and myeloid cells and functions as a negative regulator of immunity. FcγRIII is restricted to macrophages, neutrophils, natural killer cells, and mast cells. Interaction of IgG with FcγRI or FcγRIII leads to cellular activation, which requires interaction of these receptors with a homodimer of the so-called FcRγ-chain. The situation for human Fcγ receptors is more complex than the mouse system. Like mice, humans express 3 types of Fcγ receptors—a high-affinity receptor, FcγRI (CD64), and the low-affinity receptors FcγRII (CD32) and FcγRIII (CD16). However, different forms of these low-affinity receptors exist—FcγRIIA, which functions as an activating Fcγ receptor, and FcγRIIB, which is a regulatory Fcγ receptor that has an inhibitory function. In addition, different forms of the FcγRIII receptor exist in humans—FcγRIIIb, which is exclusively expressed on neutrophils, and FcγRIIIA, which is expressed on natural killer cells and macrophages. An important difference in

From the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, the Laboratory of Experimental and Clinical Immunology, and the Department of Pediatrics, Academical Medical Center, University of Amsterdam; the Department of Clinical Chemistry, Academical Hospital VU, Amsterdam; and the Department of Immunology and Genmab, University Medical Center Utrecht, The Netherlands.

Submitted November 17, 2000; accepted April 5, 2001.

Reprints: Wim K. Bleeker, CLB, Department of Immunopathology, Academical Medical Center, University of Amsterdam Plesmanlaan 125, 1066 CX, Amsterdam, The Netherlands; e-mail: w_bleeker@clb.nl.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

Fcγ receptor expression between humans and mice is the expression of FcγRIIA, which is expressed on human but not on mouse platelets.[11,12] Recently, McKenzie et al[9] demonstrated an important role for this Fcγ receptor in the induction of experimental ITP using mice overexpressing human FcRIIA.

IVIG preparations contain variable amounts of monomers and dimers and small amounts of aggregated IgG; it is unknown which of these fractions constitutes the active component, making standardization of IVIG preparations for the treatment of ITP tedious. The aim of this study was to develop a clinically relevant murine model for ITP. The usefulness of the experimental model was demonstrated by assessing the component in IVIG-mediating therapeutic efficacy, which was shown to represent the IgG dimeric fraction.

Materials and methods

Animals

Female C57BL/6 mice of 20- to 25-g body weight were obtained from Harlan CPB (Zeist, Netherlands) and kept in our animal facilities. FcRγ-chain deficient and FcγRIII-deficient mice, lacking FcγRI and FcγRIII[13,14] or FcγRIII[15] alone, respectively, were both used in a C57BL/6 background.

IVIG immunoglobulin preparations

Human γ-globulin for IVIG use was obtained from the CLB (immunoglobulin IV, 6% wt/vol; Amsterdam, The Netherlands). This is a freeze-dried product prepared from pooled plasma from at least 1000 donors by Cohn fractionation followed by pepsin incubation, pH 4. Fresh IVIG was immediately frozen after reconstitution and kept at −80°C until further use. To obtain an IVIG preparation with a high IgG dimer content, we reconstituted a freeze-dried IVIG preparation and stored it at 4°C (further referred to as aged IVIG). A gradual increase in dimer content was observed from less than 5% to values greater than 10% after 1 week of storage. The percentage of IgG dimers remained at this level when analyzed after several months or even after 1 year of storage. Monomeric IgG was prepared by gel filtration of IVIG on a TSK4000 column. Fractions corresponding to the monomeric peak were collected and concentrated to a concentration of 50 mg/mL using Centriplus concentrators according to the manufacturer's instructions (Amicon, Beverly, MA). IVIG preparations used in the experiments were analyzed for actual monomer, dimer, and polymer contents on a calibrated Superose 12 gel filtration column connected to an FPLC system (Pharmacia, Uppsala, Sweden). A computer program (Ezchrom Chromatography Data System, version 6.5) was applied to determine the peak areas of chromatograms.

Reagents

Cobra venom factor (CoVF) was kindly provided by Dr J. S. Verbeek (LUMC, Leiden, The Netherlands). Rat antimouse platelet monoclonal antibody (mAb; MWReg30, IgG1) and rat anti–mouse FcγRII/III mAb (2.4G2, IgG2b) were obtained from Pharmingen (San Diego, CA). Fluorescein isothiocyanate-conjugated $F(ab)_2$ fragments of goat anti–rat IgG were obtained from Caltag (Burlingame, CA).

Induction of thrombocytopenia

Mice were rendered thrombocytopenic by administration of the rat mAb MWReg30, directed to the platelet-specific integrin $\alpha_{IIb}\beta_3$ (gpIIb/IIIa). In an acute model, a bolus of 5 μg mAb was injected in the peritoneal cavity in 200 μL saline. The effect of IVIG was determined in mice by giving IVIG (1 g/kg) or saline intravenously 1 hour before injection of MWReg30. Blood was obtained from the retro-orbital plexus of anesthetized mice using heparinized hematocrit tubes (Clinitubes; Radiometer, Copenhagen, Denmark). Blood samples were taken before IVIG or saline treatment (t = 0), 5 minutes before injection of MWReg30 and 10, 60, and 180 minutes after injection of MWReg30. For determination of platelet numbers, blood (20 μL) was diluted in isotone and centrifuged at 300g for 10 minutes at room temperature. Numbers of platelets were counted using an electronic cell counter (model 2F and Channelyser model 256; Coulter Electronics, Dunstable, United Kingdom).

To obtain a clinically more relevant model for testing IVIG, MWReg30 was continuously infused using an osmotic pump implanted in the peritoneal cavity (Alzet micro-osmotic pump, model 1002; Alza, Palo Alto, CA). Pumps with a pumping rate of 0.25 μL/h for a duration of 14 days were filled with 100 μL mixture containing MWReg30 (165 μg/mL), human serum albumin (1.5 mg/mL to prevent absorption of proteins to the device), and an irrelevant mouse mAb directed against human CRP to check for the infusion rate. Blood samples were taken under anesthesia at t = 0, 4, 5, and 6 days to determine numbers of platelets in peripheral blood. At day 4, aged IVIG, monomeric IgG, or saline was given intravenously at a dose of 1 g/kg.

Complement-depleted mice

Complement-depleted mice were prepared by intraperitoneal injection of 2 doses of 500 U CoVF in 200 μL saline at a 6-hour interval. Sera were analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to confirm complement depletion.

Detection of anti-idiotype antibodies directed against MWReg30

To detect antibodies in the IVIG preparations with affinity to MWReg30, IVIG was added to 96-well microtiter plates (Maxisorb: Nunc, Roskilde, Denmark) coated with MWReg30 (0.8 μg/mL), human serum albumin (HSA; 1 μg/mL), or buffer. Bound antibodies were detected by an alkaline phosphatase–labeled monoclonal antibody to human IgG (clone GG-5; Sigma Chemical, St Louis, MO). After the addition of p-nitrophenyl phosphatase as substrate, the optical density was measured with Titertek Multiscan (Flow Labs, McLean, VA)

Detection of free circulating MWReg30 in plasma and platelet-bound MWReg30

For the determination of membrane binding of MWReg30 to platelets, whole blood from mice with implanted pumps was stained at a final concentration of 5 μg/mL fluorescein isothiocyanate-conjugated $F(ab)_2$ fragments of goat anti–rat IgG mAb for 30 minutes (4°C in the dark) and analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). To detect free circulating MWReg30 in plasma from thrombocytopenic mice, plasma from mice with implanted pumps was incubated for 1 hour at room temperature with whole blood from control mice. Binding of MWReg30 to platelets was assessed as described above.

Statistical analyses

Results are expressed as mean ± SEM. Statistical analyses were performed using Student *t* tests. $P < .05$ was considered significant.

Results

MWReg30 bolus injection model for ITP

First, we determined the clearance of platelets after intraperitoneal injection of a rat mAb directed against mouse platelet-specific integrin $\alpha_{IIb}\beta_3$ (gpIIb/IIIa; MWReg30), at a dose of 5 μg per mouse. After injection, platelet numbers decreased over several hours, reaching platelet counts of $1.43 \pm 0.49 \times 10^8$/mL (n = 5, 22% of control, $P < .01$) at 3 hours (Figure 1). To determine the mechanism of antibody-induced thrombocytopenia, mice were pretreated with an mAb against FcγRII/III or, alternatively, with CoVF to deplete complement. Figure 1 shows that in vivo blocking of FcγRII/III by pretreatment with the 2.4G2 mAb completely prevented the MWReg30-induced clearance

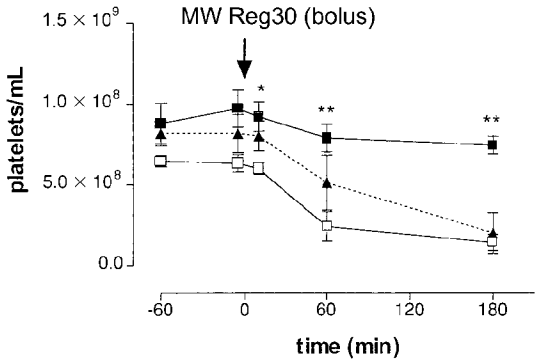


Figure 1. Intraperitoneal injection antiplatelet mAb. The role of Fcγ receptors in antibody-induced thrombocytopenia after intraperitoneal injection of 5 μg of antiplatelet mAb MWReg30. Time-course of mean platelet counts (± SEM, n = 6) is shown after injection of MWReg30 at t = 0 minute. Mice were pretreated with 2.4G2 (■, 5 μg/g intravenously), CoVF(▲), or saline (□), before MWReg30. Baseline platelet numbers were 6.37 ± 0.32 × 10^8/mL blood. Results are expressed as the mean of 3 experiments ± SEM. Asterisks indicate statistically significant differences compared to saline control (*$P < .05$; **$P < .01$).

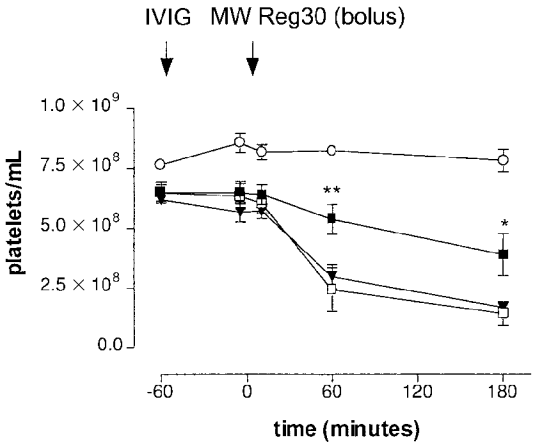


Figure 2. Effect of IVIG in antibody-mediated thrombocytopenia after intraperitoneal injection. Immune thrombocytopenia after intraperitoneal injection of antiplatelet mAb MWReg30. Time-course of mean platelet counts (± SEM) is shown after injection of MWReg30 at t = 0 minute. Mice were pretreated with aged IVIG (■), fresh IVIG (▼), or saline (□) 1 hour before MWReg30 at a dose of 1 g/kg. Injection of aged IVIG alone, without subsequent injection of the antiplatelet mAb (○), was used as a control. Results are expressed as mean of 6 experiments ± SEM on different occasions. The aged IVIG preparation used in the experiment was stored at 4°C for 1 year. Asterisks indicate statistically significant differences compared to saline control (*$P < .05$; **$P < .01$).

of platelets, whereas complement depletion did not have an effect. Analysis of sera on SDS-PAGE confirmed complement depletion. These results indicated the enhanced platelet clearance in this model to be mediated by Fcγ receptors.

Different IVIG preparations were evaluated for their effect on platelet clearance in the model. Table 1 shows the actual amounts of monomeric, dimeric, and polymeric IgG in the IVIG preparations evaluated in the ITP model. To evaluate the effect of these IgG preparations in our model, mice were pretreated with either fresh IVIG or aged IVIG at a dose of 1 g/kg, 1 hour before injection of MWReg30 (Figure 2). With intraperitoneal injection of MWReg30, the drop in platelet counts was significantly smaller in mice pretreated with aged IVIG. The final count was 3.88 ± 0.89 × 10^8/mL (60% of baseline, n = 5, $P < .05$) as measured at 3 hours. Significant differences were already observed 1 hour after the injection of MWReg30. In contrast, pretreatment of mice with fresh IVIG did not significantly decrease the effect on platelet numbers; the final count was 1.69 ± 0.23 × 10^8/mL (27% of baseline, n = 5), suggesting an important role for IgG dimers in the therapeutic effect in this model. Administration of aged IVIG in the absence of MWReg30 did not result in a change of platelet counts.

It has been reported that in vivo application of high doses of MWReg30 (greater than 30 μg intravenously) in mice induces acute hyperthermia and pathologic changes, especially in the lung, probably caused by macrophage activation leading to PAF production.[16,17] However, a recent study shows that lower doses of MWReg30 (less than 7.5 μg) do not induce pathologic changes in mice.[17] In accordance with the latter findings, we did not find any macroscopic pathologic changes in lungs at the low dose (5 μg intraperitoneally) we used to induce thrombocytopenia. Furthermore, microscopic examination of the lungs did not show signs of lung damage, such as enhanced thickness of alveolar septa, because of edema and increased cellularity (data not shown).

Table 1. Percentages of monomeric, dimeric, and polymeric immunoglobulin G in intravenous immunoglobulin preparations

	Monomeric IgG (%)	Dimeric IgG (%)	Polymeric IgG (%)
Fresh IVIG	97.6	2.7	0.5
Aged IVIG	87.2	12.0	0.8
Monomeric IgG	99.5	0.4	< 0.1

Analysis was performed by size exclusion chromatography using a calibrated Superose 12 gel filtration column connected to an FPLC system, as described in "Material and methods."

IgG indicates immunoglobulin G; IVIG, intravenous immunoglobulin.

Continuous MWReg30 infusion model

To determine the effect of IVIG in a setting better paralleling the clinical situation, MWReg30 was continuously infused using an implanted osmotic pump. After 4 days, platelet numbers reached a steady state of approximately 25% of control (Figure 3). To check the efficacy of the osmotic pumps, an irrelevant mAb, directed against human CRP, was added to the mixture of MWReg30 and HSA. The amount of this antibody was determined using an enzyme-linked immunosorbent assay in plasma samples taken at different time points during the experiment (data not shown). All mice analyzed were found to have the same amount of circulating antibody until 6 days after implantation of the pumps, indicating pumps to be equally efficient in releasing antibodies.

In the acute bolus model, we used 2.4G2 to block Fc receptors. It is known that infusion of this antibody efficiently blocks Fc receptor–mediated immune clearance; however, the maximal inhibition by this antibody only persists for 24 hours.[6] The continuous infusion model takes much longer; therefore, we decided to use Fc receptor knockout mice to avoid repeated injection of 2.4G2 in these mice. Mice deficient in the common FcRγ chain (FcRγ$^{-/-}$), lacking FcγRI and FcγRIII, and mice deficient in the FcγRIII alone were used to determine the role of the different Fcγ receptors in this model. No MWReg30-induced thrombocytopenia was observed in either FcRγ chain$^{-/-}$ or FcγRIII$^{-/-}$ mice, suggesting an important role for FcγRIII in the clearance of antibody-sensitized platelets (Figure 3A).

Administration of IVIG in patients with ITP is often associated with a rapid increase in platelet counts. To determine whether administration of IVIG shows a similar effect on platelet counts in our model, mice were treated with aged IVIG or monomeric IgG 4 days after implantation of the pumps. To better determine the role of IgG dimers, we used in this series a monomeric IgG fraction isolated from IVIG instead of fresh IVIG because the latter still contains a significant amount of IgG dimers (Table 1). Infusion of a

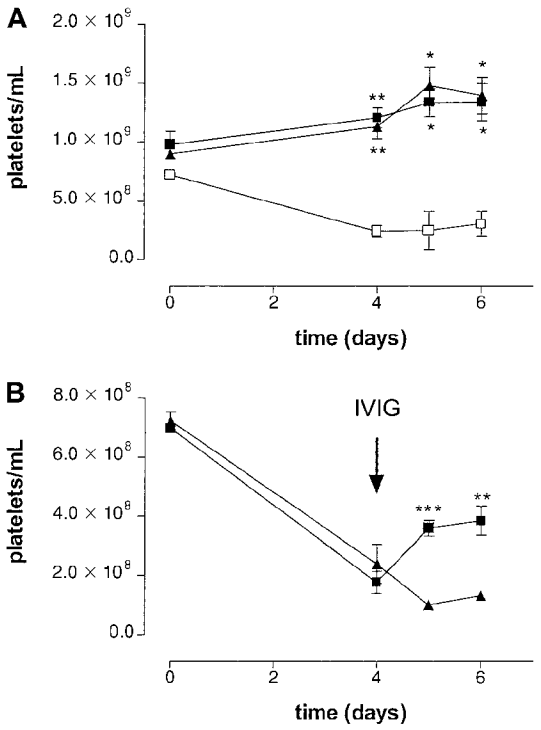


Figure 3. Effect of IVIG in antibody-mediated thrombocytopenia after continuous infusion of antiplatelet mAb. (A) Effect on platelet counts by continuous infusion of antiplatelet mAb MWReg30 by an implanted osmotic pump in wild-type (□), FcγRIII$^{-/-}$ (■), and FcR γ-chain$^{-/-}$ (▲) mice. Time course of mean platelet counts of 3 experiments (± SEM) is shown except for the wild-type mice (n = 2). Asterisks indicate statistically significant differences compared to saline control (*$P < .05$; **$P < .01$). B) Effect on platelet counts after intravenous injection of aged IVIG (■) or monomeric IgG (▲) at t = 4 days. Aged IVIG preparations used in the experiment was stored at 4°C for 1 year. Results are expressed as mean of 5 experiments ± SEM on different occasions. Asterisks indicate statistically significant differences between the 2 groups (**$P < .01$; ***$P < .001$).

single dose of aged IVIG resulted in a transient increase in platelets for several days (Figure 3B), reaching platelet counts of approximately 52% of baseline values. No increase in platelet counts was observed after infusion of monomeric IgG or saline.

Table 2 shows the results of FACS analysis for detecting MWReg30 bound to platelets (membrane bound) and circulating amounts of MWReg30 in plasma expressed as mean fluorescence intensity. We tested whether circulating MWReg30 could be detected in plasma with continuous infusion and after bolus injection. Serial dilution of MWReg30 incubated with control platelets was used as a reference to estimate the amount of MWReg30 in plasma with continuous infusion. At day 4, 0.89 μg/mL anti-CRP was present in the plasma, indicating a 125-fold dilution of the input (112 μg/mL). Based on the amount of anti-CRP mAb measured in the enzyme-linked immunosorbent assay, we also expected a 1:125 dilution of MWReg30 to be present in plasma at day 4 if no antibody bound to membrane surfaces. Table 2 shows that binding of antibody in plasma was similar to a 1:2500 dilution of the infused MWReg30 solution, suggesting that approximately 5% of MWReg30 was not bound to platelets. Furthermore, membrane binding of MWReg30 in vivo was shown to be one third the maximal value for each type of administration.

Anti-idiotypic antibodies against MWReg30 in IVIG

IVIG efficacy in ITP could also result from the neutralization of platelet antibodies by anti-idiotypic antibodies in IVIG. Hence, aged IVIG and fresh IVIG were evaluated for the presence of anti-idiotypic antibodies with binding capacity to MWReg30. As a control, we tested possible binding capacity of components present in the IVIG preparations to human serum albumin. Binding of IgG to different coatings was similar: no specific binding to MWReg30 was observed when testing the various preparations (data not shown). We also evaluated whether IVIG could interfere with the binding of MWReg30 to platelets. Aged IVIG, at a concentration of 5 mg/mL, did not reduce MWReg30 binding of murine platelets in vitro.

Discussion

In the present study, we used 2 murine models for ITP to evaluate the effect of IVIG preparations that differed in the content of IgG dimers. We demonstrated that clearance of platelets sensitized with an antiplatelet mAb could only be reduced by administration of an IVIG preparation containing significant amounts of IgG dimers. An IVIG preparation without IgG dimers hardly showed an effect. The effect of IgG dimers was observed in both murine models for ITP, that is, a single intraperitoneal injection or continuous infusion of antiplatelet antibody. We recently described the hypotensive effects of IgG dimers in IVIG preparations, in which we compared 16 different IVIG preparations of 11 different manufacturers.[18] This study revealed that commercially available IVIG preparations contain variable amounts of IgG dimers (range, 5%-15%). This is consistent with the observations of Tankersley et al[19] that IgG dimers are a normal constituent of IVIG prepared from a large donor pool. The amount of dimers in the preparations mainly depends on the pool size and the storage conditions. Our present findings strongly suggest that because of differences in dimer content, preparations also will differ in therapeutic efficacy in the treatment of patients with ITP. They also suggest that therapeutic efficacy in ITP entails the risk for side effects, probably also related to the presence of IgG dimers through the activation of macrophages and neutrophils.[18,20]

Low-affinity Fcγ receptors apparently were involved in the antibody-dependent clearance of platelets in our model because in vivo blocking of FcγRII/III or the absence of these receptors in knock-out mice completely prevented the effect of antiplatelet mAb. The importance of low-affinity Fcγ receptors in the clearance of immune complexes has been shown in several human and animal studies.[6] Furthermore, infusion of mAbs against FcγRIII caused a significant, transient increase in platelet counts in a patient with ITP.[10] Infusion of an mAb directed against FcγRI did not change the platelet count over a 5-day course of treatment, whereas the administration of IVIG in this patient improved platelet count.[21] Our experiments with blocking mAb (2.4G2) or in mice deficient for Fcγ receptors were compatible herewith and in particular pointed to a role for FcγRIII in the clearance of antibody-sensitized

Table 2. Platelet-bound and free circulating MWReg30 in the thrombocytopenic mice

	Platelet-bound MWReg30 (MFI)
Circulating platelets	
Osmotic pump (n = 3)	700 ± 150
Bolus (n = 3)	683 ± 111
Normal platelets incubated with plasma	
Osmotic pump (n = 3)	51 ± 13
Bolus (n = 3)	27 ± 1
Normal platelets incubated with MWReg30	
1:100	2266 ± 634
1:500	501 ± 299
1:2500	58 ± 10
control	18 ± 9

MFI indicates mean fluorescence intensity.

platelets, emphasizing the relevance of these models for human ITP. However, others have demonstrated involvement of the platelet Fcγ receptor in human immune thrombocytopenias accompanied by thrombosis, such as heparin-induced thrombocytopenia,[9] and it cannot be precluded that this receptor also plays a pathophysiologic role in other forms of ITP. Because mice do not express this Fcγ receptor on platelets, our model is unable to address beneficial or detrimental effects of IVIG on platelet Fcγ receptors. Hence, one should be careful to extrapolate the results obtained in this model to human ITP. Interference with FcγR expression level and FcγR-mediated functions (such as phagocytosis) are well-established characteristics of polymeric IgG and immune complexes.[22,23] Furthermore, human IgG aggregates and IgG dimers bind more efficiently to low-affinity Fcγ receptors than do IgG monomers. Dimeric IgG may, therefore, have a stronger effect than monomeric IgG. It has been suggested that in IVIG, small amounts of aggregated IgG can form spontaneously and that these aggregates, rather than IgG monomer itself, constitute the active component of IVIG mediating Fcγ-receptor blockade in ITP.[7,19] Augener[24] reported that the efficacy of IVIG treatment of a patient with ITP was not associated with monomeric IgG levels but with the presence of IgG aggregates in the preparation. This patient showed normal levels of IgG complexes before treatment with IVIG and showed stepwise increases in total IgG levels, including aggregates, during therapy. When IVIG administration was terminated, the concentration of complexed IgG rapidly dropped to a normal level, and this was paralleled by a drastic decrease of platelet numbers even though IgG plasma levels were still high.[24] In another study it was shown that the use of intramuscular preparations of anti-D that contained aggregated IgG resulted in small platelet increases in several Rh-negative recipients.[1] These observations agree well with the results of our study.

Acute systemic response seen on a bolus injection of a relatively high dose of MWReg30 is a process that requires a threshold-dose of antibody to occur. The density of antibodies bound to the platelets can explain these differences. In patients with ITP, platelet-specific antibodies are primarily directed to gpIIb/IIIa—as is MWReg30. However, specific antiplatelet antibodies account for only a small fraction of the platelet-associated IgG, with the major fraction attributed to nonspecific IgG associated with platelets.[25,26] This suggests that in a model with clinical relevance, the amount of antibody used to induce thrombocytopenia should be low, so that only the antibody occupies a few epitopes on the platelet surface. We showed that in our models only some of the MWReg30 binding sites were occupied.

An alternative mechanism for the reversal of thrombocytopenia by IVIG involves idiotype–anti-idiotype interaction with autoantibodies and anti-idiotype antibodies in the IVIG preparations.[5] This mechanism is independent of RES blockade, but it postulates the neutralization of antiplatelet antibody by IVIG. However, it was shown that Fcγ fragments have the same therapeutic effects as IVIG in patients with ITP.[27] Thus, anti-idiotypic effects of IVIG are not likely a major mechanism of action in ITP. In our study, binding of MWReg30 to platelets was not influenced by IVIG, nor did we find (anti-idiotypic) antibodies with binding capacity to MWReg30.

In conclusion, in a clinically relevant mouse model for ITP, we show the efficacy of IgG preparations containing high amounts of IgG dimers. The experimental model applied may not only help to define the mechanisms causing ITP but may also add to the evaluation of novel therapeutic strategies to treat this disease. We suggest that therapeutic efficacy of different IVIG preparations used to treat human ITP depends on the presence of IgG dimers, with preparations containing relatively high amounts of IgG dimers having better therapeutic efficacy.

References

1. Bussel J. Fc receptor blockade and immune thrombocytopenic purpura. Semin Hematol. 2000;37:261-266.
2. Imbach P, Barandun S, dÁpuzzo V. High-dose intravenous gamma-globulin for idiotypic thrombocytopenia purpura in childhood. Lancet. 1981; 1:1228-1231.
3. Blanchette V, Carcao M. Intravenous immunoglobulin G and anti-D as therapeutic interventions in immune thrombocytopenic purpura. Transfus Sci. 1998;19:279-288.
4. Mouthon L, Kaveri S, Kazatchkine M. Immune modulating effects of intravenous immunoglobulin (ivIG) in autoimmune diseases. Transfus Sci. 1994;15:393-408.
5. Lazarus A, Freedman J, Semple J. Intravenous immunoglobulin and anti-D in idiotypic thrombocytopenic purpura (ITP): mechanisms of action. Transfus Sci. 1998;19:289-294.
6. Kurlander R, Ellison M, Hall J. The blockade of Fc receptor-mediated clearance of immune complexes in vivo by a monoclonal antibody (2.4G2) directed against Fc receptors on murine leukocytes. J Immunol. 1984;133:855-862.
7. Kurlander R, Hall J. Comparison of intravenous gamma globulin and a monoclonal anti-Fc receptor antibody as inhibitors of immune clearance in vivo in mice. J Clin Invest. 1986;77:2010-2018.
8. Sylvestre D, Clynes R, Ma M, Warren H, Carroll M, Ravetch J. Immunoglobulin G-mediated inflammatory responses develop normally in complement-deficient mice. J Exp Med. 1996; 184:2385-2392.
9. McKenzie S, Taylor S, Malladi P, et al. The role of a human Fc receptor FcgRIIA in the immune clearance of platelets: a transgenic mouse model. J Immunol. 1999;162:4311-4318.
10. Clarkson S, Bussel J, Kimberly R, Valinsky J, Nachman R, Unkeless J. Treatment of refractory immune thrombocytopenic purpura with an anti-Fcγ-receptor antibody. N Engl J Med. 1986;314: 1236-1239.
11. Ravetch J, Kinet J. Fc receptors. Annu Rev Immunol. 1991;9:457-492.
12. Ravetch J. Fc receptors. Curr Opin Immunol. 1997;9:121-125.
13. van Vugt M, Heijnen I, Capel P, et al. FcR γ-chain is essential for both surface expression and function of human FcγRI (CD64) in vivo. Blood. 1996; 87:3593-3599.
14. Park S, Ueda S, Hamano Y, et al. FcR γ-chain–deficient mice are resistant to glomerulonephritis. J Clin Invest. 1998;102:1229-1238.
15. Hazenbos W, Gessner J, Hofhuis F, et al. Impaired IgG-dependent anaphylaxis and Arthus reaction in FcγRIII (CD16)-deficient mice. Immunity. 1996;5:181-188.
16. Nieswandt B, Echtenacher B, Wachs F, et al. Acute systemic reaction and lung alteration induced by an antiplatelet integrin gpIIb/IIIa antibody in mice. Blood. 1999;94:684-693.
17. Nieswandt B, Bergmeier W, Rackebrandt K, Gessner J, Zirngibl H. Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice. Blood. 2000;96:2520-2527.
18. Bleeker W, Teeling J, Verhoeven A, et al. Vasoactive side effects of intravenous immunoglobulin preparations in a rat model and their treatment with recombinant platelet-activating factor acetylhydrolase. Blood. 2000;95:1856-1861.
19. Tankersley D, Preston M, Finlayson J. Immunoglobulin G dimer: an idiotypic-anti-idiotypic complex. Mol Immunol. 1988;22:41-48.
20. Teeling J, Bleeker W, Rigter G, van Rooijen N, Kuijpers T, Hack C. Intravenous immunoglobulin preparations induce mild activation of neutrophils in vivo via triggering of macrophages: studies in a rat model. Br J Haematol. 2001;112:1031-1041.
21. Ericson S, Coleman K, Wardell K, et al. Monoclonal antibody 197 (anti-FcγRI) infusion in a patient with immune thrombocytopenia purpura (ITP) results in down-modulation of FcγRI on circulating monocytes. Br J Haematol. 1996;92:718-724.
22. Heijnen I, van de Winkel J. Human IgG Fc receptors. Int Rev Immunol. 1997;16:29-56.
23. Ravetch J. Fc receptors: rubor redux. Cell. 1994; 78:553-560.
24. Augener W, Friedman B, Brittinger G. Are aggregates of IgG the effective part of high-dose immunoglobulin therapy in adult idiotypic thrombocytopenic purpura (ITP)? Blut. 1985;50:249-252.
25. Chong B, Keng T. Advances in the diagnosis of idiopathic thrombocytopenic purpura. Semin Hematol. 2000;37:249-260.
26. Kelton J, Steeves K. The amount of platelet-bound albumin parallels the amount of IgG on washed platelets from patients with immune thrombocytopenia. Blood. 1983;62:924-927.
27. Debré M, Bonnet M, Fridman W, et al. Infusion of Fcγ fragments for treatment of children with acute immune thrombocytopenic purpura. Lancet. 1993;342:945-949.